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The Clonal Origin of Myocardial Cells in Different Regions of the Embryonic Mouse Heart

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Summary

When and how cells form and pattern the myocardium is a central issue for heart morphogenesis. Many genes are differentially expressed and function in subsets of myocardial cells. However, the lineage relationships between these cells remain poorly understood. To examine this, we have adopted a retrospective approach in the mouse embryo, based on the use of the *laacZ* reporter gene, targeted to the α-*cardiac actin* locus. This clonal analysis demonstrates the existence of two lineages that segregate early from a common precursor. The primitive left ventricle and the presumptive outflow tract are derived exclusively from a single lineage. Unexpectedly, all other regions of the heart, including the primitive atria, are colonized by both lineages. These results are not consistent with the prespecification of the cardiac tube as a segmented structure. They are discussed in the context of different heart fields and of the evolution of the heart.

Introduction

The adult heart in birds and mammals is composed of integrated functional units, which pump the oxygenated and deoxygenated blood separately in a unidirectional flow. The cardiac chambers (ventricles and atria) have distinct properties and are specifically connected to one another or to the great vessels by valves. These units are prefigured, prior to septation and valve formation, by regions of the embryonic heart tube, which are organized in a linear array from the arterial to the venous pole, as presumptive outflow tract, embryonic right ventricle, embryonic left ventricle, atrio-ventricular canal, right and left atria, and inflow tract (de la Cruz et al., 1989; see Moorman et al., 2003). They can be distinguished morphologically, from the time (embryonic day [E] 9.5 in the mouse) when the chambers expand outwards from the tube, which undergoes looping, leading to the juxtaposition of the arterial and venous poles. The embryonic regions express specific molecular markers, and mutations in some of these genes affect the formation of particular cardiac chambers, although mechanistically this is poorly understood (Harvey, 2002). The acquisition of regional identity is also associated with characteristic cell behavior, as indicated by the specific orientation of clonal growth in the early myocardium (Meilhac et al., 2004).

When and how does cardiac regional identity emerge during embryonic development? Is this potential inherited within a prespecified lineage or acquired once cells have assumed their final position, as a result of environmental induction? It is mainly in the chick embryo, which is amenable to experimental manipulation, that the degree of regionalization of myocardial precursor cells has been examined, before they differentiate and are incorporated into the cardiac tube. Based on grafting experiments, it has been proposed that myocardial precursor cells in the primitive streak, at the time of gastrulation, have a rostro-caudal organization (Garcia-Martinez and Schoenwolf, 1993), which is maintained during their migration rostrally (Stalsberg and De Haan, 1969) and prefigures the arterial-venous regionalization of the cardiac tube. It was thus concluded that the heart tube is prespecified as a segmented structure, where segments correspond to future functional units (de la Cruz et al., 1989; see Brand, 2003). However, DiI labeling experiments have challenged the view that this occurs very early, since no regionalization of myocardial precursor cells has been detected until the cardiac crescent stage, just before heart tube formation (Redkar et al., 2001). It is also only at this stage that the perturbation of rostrocaudal organization of cardiac precursor cells by grafts leads to abnormal heart morphogenesis (Patwardhan et al., 2000). Consistent with these results, we have shown in the mouse that myocardial precursor cells undergo an early phase of dispersive growth (Meilhac et al., 2003).

Other subdivisions of the myocardium have been proposed, based on the embryonic origin of precursor cells and on the time at which they are incorporated into the cardiac tube. The cardiac tube forms initially as a result of fusion of the cardiac crescent, which already contains differentiated myocardial cells. It has been shown that precursor cells continue to be incorporated into the poles of the heart, particularly posteriorly into the venous pole (Viragh and Challice, 1973). More recently, it has been demonstrated that pharyngeal mesoderm is also a source of myocardial cells, both in birds (Waldo et al., 2001; Mjaatvedt et al., 2001) and mammals (Kelly et al., 2001). This anterior or secondary heart field later contributes cells to the arterial pole, where they form the outflow tract, as shown by transgenic markers and DiI labeling. In the mouse, this field is already distinguishable at E7.5; however, its clonal relationship with the cardiac crescent or primary heart field remains unclear, as does the extension and overlap of their respective contribution to the cardiac tube.

Another view of cardiogenesis, based on morphological, electrophysiological, and molecular data, stresses the distinction between the myocardium of the primitive heart tube, which retains its characteristics later as the inflow and outflow tracts and atrio-ventricular canal, and the cardiac chambers (atria and ventricles), which bulge

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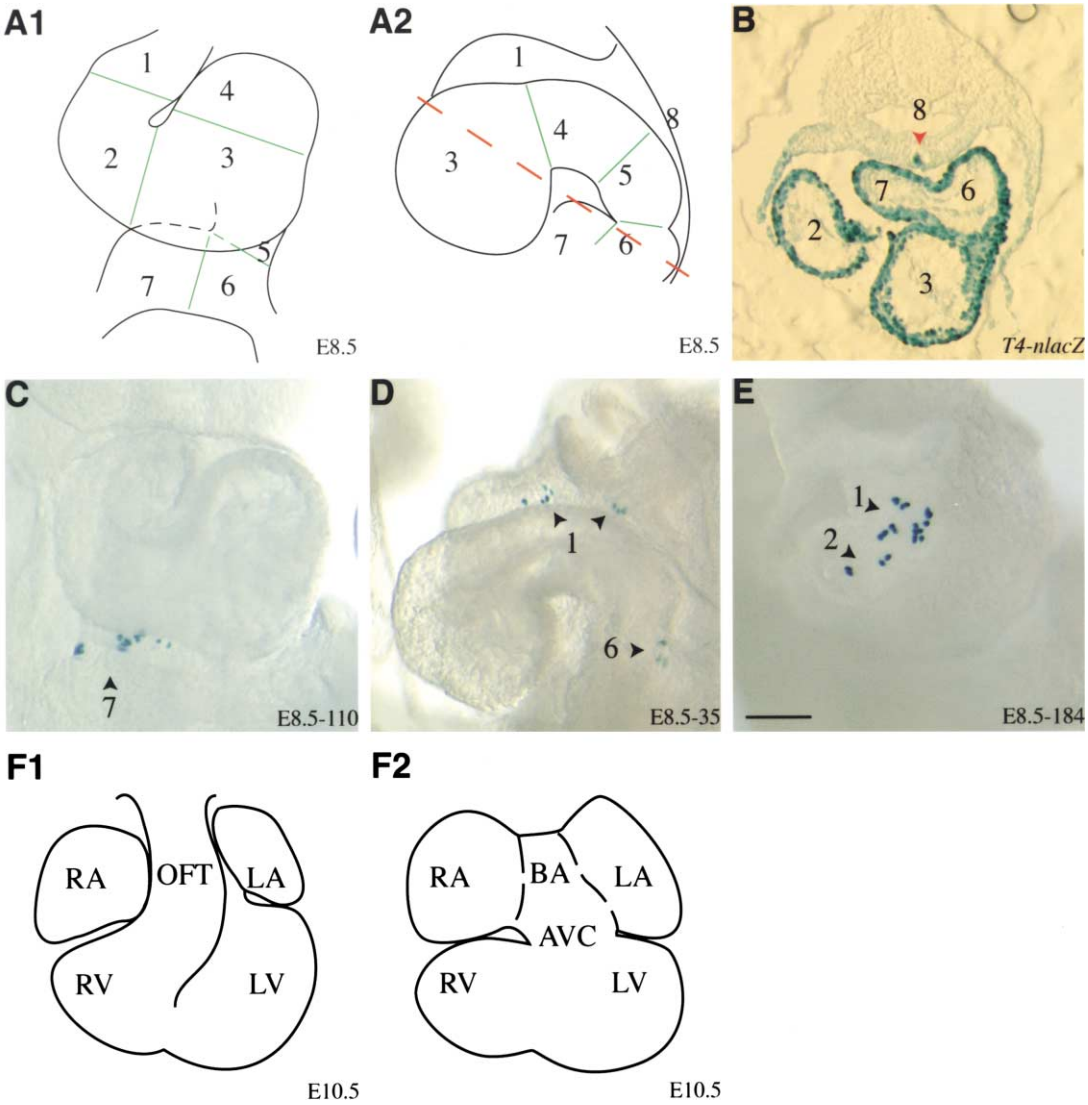

Figure 1. Subdivision of the Arterial-Venous Axis of the E8.5 Cardiac Tube

(A) Localization of the regions (numbered 1–8) used to define the organization of clones along the arterial-venous axis of the cardiac tube at E8.5, in ventral (A1) and left lateral (A2) views. Black and green dotted lines indicate tissue lying behind the structures presented in A1.

(B) Transverse section, at the level indicated by a dotted red line in A2, showing X-gal staining throughout the myocardium of an E8.5 embryo from the transgenic mouse line *T4-nlacZ*. The red arrowhead points to the dorsal mesocardium.

(C–E) Examples of α-*cardiac actin*$^{nlaacZ1.1/+}$ hearts at E8.5 with X-gal-positive staining. Each blue dot represents a single nucleus since the reporter gene contains a nuclear localization signal. Numbered arrowheads indicate the regions in which β-galactosidase-positive cells are observed. (C) and (E) are ventral and (D) left lateral views. In all photographs (also in other figures), the pericardium has been removed and the numbers in the bottom right corner indicate the stage followed by the identification number of the embryo. Scale bar: 250 μm.

(F) Superior views of a late embryonic heart at E10.5. The outflow tract has been removed in F2 for better visualization of the underlying structures. OFT, outflow tract; RV, right ventricle; LV, left ventricle; AVC, atrio-ventricular canal; BA, body of the atrium; LA, left atrium; and RA, right atrium, potentially corresponding to regions 1–7, respectively.

out from the tube to constitute a working myocardium (Christoffels et al., 2000). Again, the lineage relationship between these myocardial subdivisions is not known.

In order to gain insight into the regionalization of the myocardium, we have investigated the lineage relationships between different parts of the heart, using retrospective clonal analysis in the mouse embryo. This is based on the spontaneous mitotic recombination, at low frequency, of an inactivated *nlaacZ* reporter sequence, to give a functional *nlacZ* sequence (Bonnerot and Nicolas, 1993). We have integrated the *nlaacZ* reporter gene into the mouse α-*cardiac actin* gene, which is expressed throughout the myocardium, so that recombination of *nlaacZ* will result in β-galactosidase-positive heart cells (Meilhac et al., 2003). This genetic approach permits the production of random clones of myocardial cells, which we analyze in detail in the murine cardiac tube at E8.5. Two distinct categories of clones are observed, designated first and second myocardial cell lineages, on the basis of their contribution to the more primitive or more recently formed part of the cardiac tube. They reflect some of the characteristics of the primary and anterior

heart fields, respectively. Clones from the first lineage never contribute to the arterial pole region and are polarized, in accordance with the oriented elongation of the cardiac tube toward the venous pole. Strikingly, clones derived from the second lineage participate not only in the outflow tract and the right ventricular region, but also in the venous pole (atrio-ventricular canal and atria) and the dorsal mesocardium. The first and second lineages have overlapping contributions to the right ventricle and the venous pole. Our analysis demonstrates a common clonal origin for the two lineages, with an apparently earlier segregation of the first lineage.

Results

In order to analyze the distribution of clones in the presumptive functional units of the heart, aligned along the arterial-venous axis of the cardiac tube, this axis was subdivided into seven consecutive regions, numbered from the arterial to the venous ends (Figure 1A). This was based on morphological criteria and follows the constrictions already evident at embryonic day (E) 8.5, as the heart loops. Based on cell behavior (Meilhac et al., 2004), and the subsequent morphogenesis of the tube (Moorman et al., 2003), regions 1–7 correspond approximately to the future proximal outflow tract (1), embryonic right ventricle (2), embryonic left ventricle (3), atrio-ventricular canal (4), body of the atrium (5), and left (6) and right atria (7), clearly distinguishable later at E10.5 (Figure 1F). An additional eighth region was colonized by clones at E8.5 and corresponds to the dorsal mesocardium, which remains localized behind the heart tube, as it closes dorsally. This is also a site of α-*cardiac actin* transcription, as visualized (Figure 1B) with a transgenic line, in which the *nlacZ* reporter is controlled by 9 kb of α-*cardiac actin* regulatory sequences (*T4-nlacZ*; Biben et al., 1996). At E8.5, 238 hearts with β-galactosidase-positive cells were obtained, out of a total of 3629 embryos dissected from the α-*cardiac actin*$^{nlaacZ1.1/+}$ line. Statistical analysis indicates that at this stage labeled cells are clonally related, with a low probability that two independent recombination events have occurred in the same heart ($<$4%, see Experimental Procedures). As described previously (Meilhac et al., 2003), β-galactosidase-positive cells are dispersed along the arterial-venous axis of the cardiac tube and form separate clusters. Restricted clones are observed, which contribute to a single region (Figure 1C). Other clones contribute to more than one region. Their distribution may be discontinuous (Figure 1D) or they may be continuous (Figure 1E), colonizing adjacent regions.

Three Categories of Clones Participate in the Arterial-Venous Axis of the Cardiac Tube

Two clonal parameters have been scored: the number of labeled cells and the extension of the clone (Figure 2). The number of labeled cells gives an indication of the date of labeling of the clone, since growth of myocardial cells and their precursors follows a proliferative mode (Meilhac et al., 2003), provided that there is a constant overall growth rate (Sissman, 1966) with negligible apoptosis (Fisher et al., 2000). The degree of regionalization, which reflects the spatial potential of the labeled precursor cell, is presented as the extension of the clone along the axis, without this necessarily being continuous. For example, a clone with labeled cells in region 1 and region 6 has an extension of 6 (Figure 1D). For the calculation of the extension, regions 6 and 7—presumptive atrial regions—were pooled, since they form the two arms of the inverted Y-shaped heart tube (Figure 1A1) and therefore correspond to the same axial level. Region 8 (dorsal mesocardium) is shown but was not taken into account for the calculation of the extension, since it is located outside the tube. In addition, regions with one to two labeled cells (asterisks in Figure 2) were not taken into account in the calculation of the extension of small clones, since statistical analysis suggests that they may represent a recent secondary labeling event (see Experimental Procedures).

It is striking that in the clones with an extension of 3–5 (Figure 2A2), there is a lack of participation in region 1 and that, in contrast, most of the clones with an extension of 6 contribute only to the extremities of the heart tube. This tendency toward a mutually exclusive distribution suggests a different clonal origin. Region 1 is colonized by two categories of large clones. Most of these (in red Figure 2B) do not contribute to region 3, with the exception of the largest clones (E8.5-198, E8.5-2 and E8.5-170, in stripes) which constitute another category referred to as "very large clones." At the other extremity of the cardiac tube, region 6/7 is colonized by the same clones (in red and in stripes), as well as by additional large clones (in black). These additional clones (in black) contribute to regions which are distinct from those colonized by the clones represented in red, namely region 3 or both regions 4 and 5. They therefore constitute a distinct category. Clones E8.5-238 and E8.5-177 have been included in the red category, because they contribute to a subset of the regions colonized by the large clones E8.5-191 and E8.5-127. Similarly, clones E8.5-215 and E8.5-102 are included in the black category, because they contribute to a subset of the regions colonized by large clones E8.5-156, E8.5-166, E8.5-237, and E8.5-25. Such subclones are a classic feature of retrospective clonal analysis, since clones may arise at any time from an ancient precursor or its descendents, giving rise to a complete clonal pattern or part of it.

In conclusion, we have classified clones based on the striking observation that regions 1 and 3 are exclusive in all but the three very large clones. We identify three categories of large clones (in red, black, or red and black stripes Figure 2B), which colonize the arterial-venous axis of the cardiac tube differently and therefore arise from precursor cells with distinct regional potential. The cells of large clones contributing to region 6/7 and not to region 1, or those of their subclones, will be referred to as the "first lineage" (in black), because of their contribution to the most primitive part of the tube. In contrast, the cells of large clones, which always contribute to region 1, or those of their subclones, will be referred to as the "second lineage" (in red). The very large clones represent a third category; they contribute to both region 1 and region 3 and have the characteristics of both

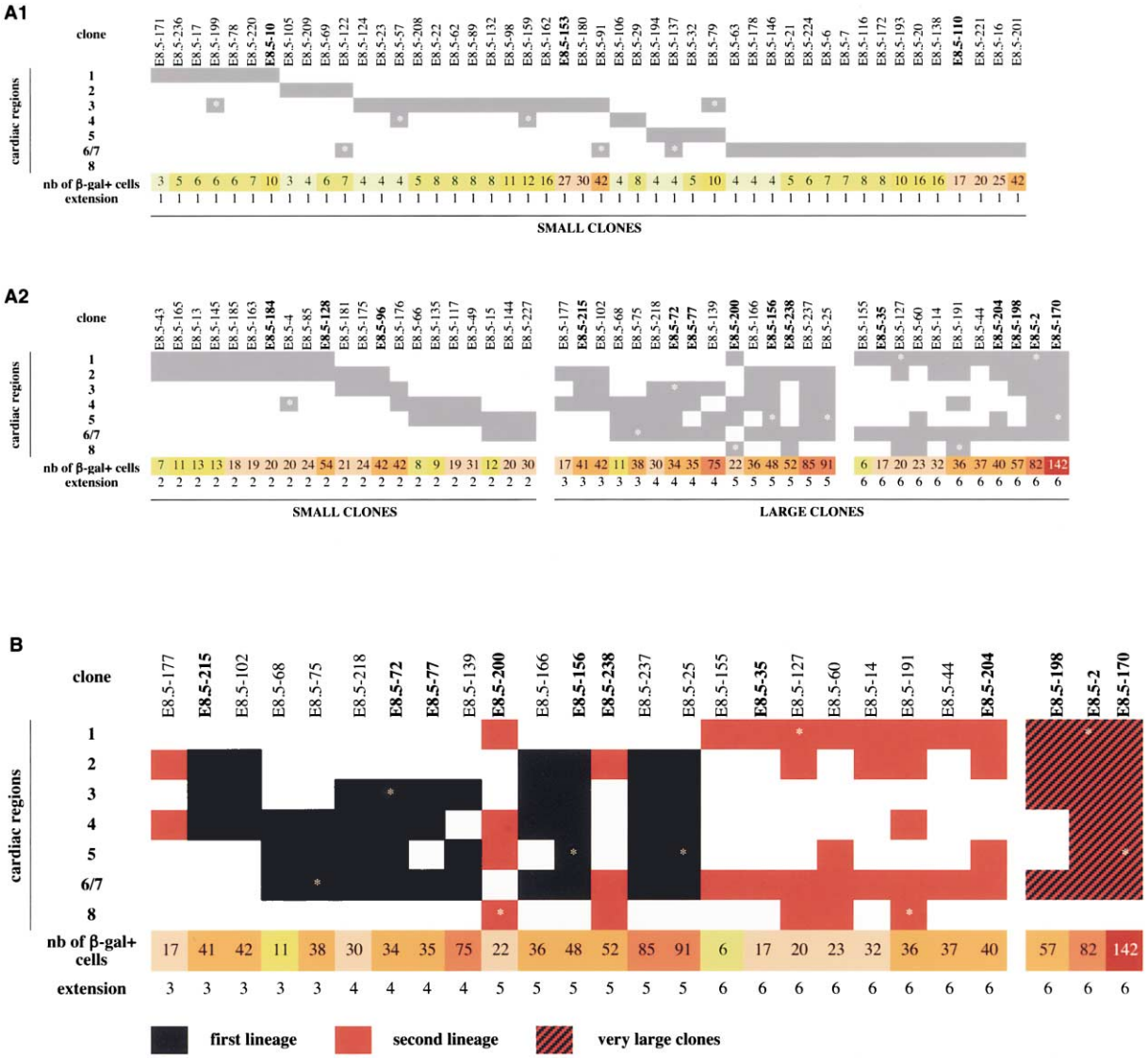

Figure 2. Schematic Representation of the Participation of Clones on the Arterial-Venous Axis of the Cardiac Tube at E8.5

(A) The distribution of β-galactosidase-positive cells in 94 hearts, containing more than two cells at E8.5, from embryos with 9–17 somites at the looped cardiac tube stage. The participation of a clone (column) in a region (line) is represented by a colored box. Clones have been classified based on their extension along the arterial-venous axis (region 8 not included, see text). Within each category of extension, clones are presented in the order of their arterial limit and of their total number of labeled cells. The number of β-galactosidase-positive cells per heart is color coded to show the exponential increase of clone size after each cell division (from light yellow to brown, 3–4, 5–8, 9–16, 17–32, 33–64, 65–128, 129–256 cells). Asterisks indicate 1–2 β-galactosidase-positive cells per region. Bold numbers indicate the clones which are illustrated by photographs in other figures.

(B) Representation of the contribution to the arterial-venous axis of the cardiac tube of three categories of large clones. We refer to clones represented in black as the first lineage and to clones in red as the second lineage. These clones have a mutually exclusive participation in the heart tube. Very large clones, which contribute to both region 3, specific to the first lineage, and region 1, specific to the second lineage, are represented in black and red stripes. Note that small clones can be unambiguously attributed to the first or second lineage only if they participate, respectively, in region 3 or 1.

lineages. Statistical tests support this classification (Table 1). The remaining clones, with an extension of 1–2, will be referred to as "small clones" (Figure 2A with examples shown in Figures 3A and 3B and Figures 4A and 4B). They very probably constitute subclones of the larger clones of each lineage; i.e., they derive from more recent precursors, because they are more frequent and contain a smaller number of cells.

Characterization of Large Clones from the First Myocardial Lineage

Examples of clones at E8.5 from the first lineage, which do not colonize the future outflow tract region at the arterial pole of the cardiac tube (region 1), are shown in Figures 3A–3F. These clones usually encompass region 3, the primitive left ventricle. Dispersion of labeled cells on the arterial-venous axis is mainly continuous, with

Table 1. Existence of Large Clones

Is a large clone from the first lineage the result of a double recombination event in more recent precursors?						
	Large clones from the first lineage[a]		Smaller clones not in region 1[a]		Expected number of double recombination events[b]	χ^2 (1 d.f.)[b]
	E = 4 or 5	E = 3	E = 2 or 3	E = 1, 2		
nb	8		16		0.07	891[c]
nb		4		51	0.72	15[c]
Is a large clone from the second lineage the result of a double recombination event in more recent precursors of a small arterial clone and of a small venous clone?						
	Large clones from the second lineage[a]	Small clones in arterial regions 1, 2[a]	Small clones in venous regions 4, 5, 6/7[a]		Expected number of double recombination events[b]	χ^2 (1 d.f.)[b]
nb	11	21	29		0.17	699[c]
Is a very large clone the result of a double recombination event in a precursor of a clone from the first lineage and in a precursor of a clone from the second lineage?						
	Very large clones[a]	Large clones from the first lineage or small clones in region 3[a]	Large clones from the second lineage or small clones in region 1[a]		Expected number of double recombination events[b]	χ^2 (1 d.f.)[b]
nb	3	30	28		0.23	33[c]

E, extension; nb, number
[a] See Figure 2.
[b] See Experimental Procedures.
[c] $p < 0.001$, therefore we conclude that this is not the case.

colonization of adjacent regions (in black Figure 2B; Figures 3B–3F). While some clones are restricted to region 3 (Figure 3A), others extend toward the arterial pole into region 2 (Figures 3B, 3C, and 3F1). However, the majority of clones extend toward the venous pole (Figures 3C–3F), from region 2, 3, or 4 (Figure 2B). Thus, clones from the first lineage appear to be polarized, with a preferential contribution to the venous region 6/7 of the cardiac tube. Polarization of clones probably reflects the polarized growth of cells during elongation of the cardiac tube. In Figures 3G and 3H, two examples of β-galactosidase-positive cells in hearts at E10.5 are shown. These have a distribution similar to that shown in Figures 3C and 3F, respectively, in accordance with the predicted cardiac domains to which regions 1–7 will contribute. Such clones at E10.5 which contribute to the left ventricle and not to the outflow tract, also show a continuous extension and a tendancy to be polarized toward the venous pole. The similar properties of clones at E10.5 indicate that regionalization of myocardial cells on the arterial-venous axis of the heart at E8.5 is maintained during embryonic development.

Characterization of Large Clones from the Second Myocardial Lineage

Figure 4 (A–E) shows examples of clones at E8.5 from the second lineage, which contribute to the arterial pole of the heart. Most of these clones colonize region 1, which corresponds to the future outflow tract (Figures 4A–4D). Some also colonize region 2 (Figures 4B, 4D, and 4E), the presumptive right ventricle. Clones from the second lineage never participate in the primitive left ventricle (region 3), and it is striking that large clones are always discontinuous (in red Figure 2B), contributing also to future atrio-ventricular and atrial regions at the venous pole of the heart (Figures 4C–4E). Labeled cells of the second lineage are more frequently localized in the inner curvature of the cardiac tube (Figures 4C and 4E) than those from the first lineage (Figures 3A–3F). It is only in this lineage that β-galactosidase-positive cells can be detected in the dorsal mesocardium (region 8 in Figure 2B and red arrowheads in Figure 4E1). Indeed, this tissue is in physical continuity with the inner curvature at early stages, before the heart tube closes dorsally (red arrowhead in Figure 1B). Therefore, a probable explanation for the discontinuity of the clones from the second lineage is that they derive from a population of precursor cells located in the splanchnic mesoderm behind the heart tube, which is initially in continuity with the inner curvature and later with the two poles of the cardiac tube. At E10.5, a similar distribution of labeled cells has been observed. Labeling is present at both extremities of the cardiac tube (Figure 4F1) or in the inner curvature, in continuity from the atrio-ventricular canal to the right ventricle (Figure 4G), without participation in the left ventricle (Figures 4F1 and 4G). Additional labeling is detected at E10.5 in a few cells in the branchial arches (four cases, as an example see white arrowhead in Figure 4F2), in continuity with that in the outflow tract (scattered cells are detected in the intervening region, data not shown). The level of β-galactosidase activity in the branchial arches is low, suggesting that differentiation into myocardial cells has just been initiated. This observation is consistent with the embryological origin of cells in the outflow tract from pharyngeal mesoderm (Kelly et al., 2001).

Overlapping Fate of Precursor Cells from the First and Second Myocardial Lineages

Clones from the first lineage, as defined above, participate in regions 2 to 6/7 (in black Figure 2B), whereas those from the second lineage participate in regions 1 and 2 and 4 to 8 (in red Figure 2B). Regions 2, 4, 5, and 6/7 are therefore formed from both categories of clones

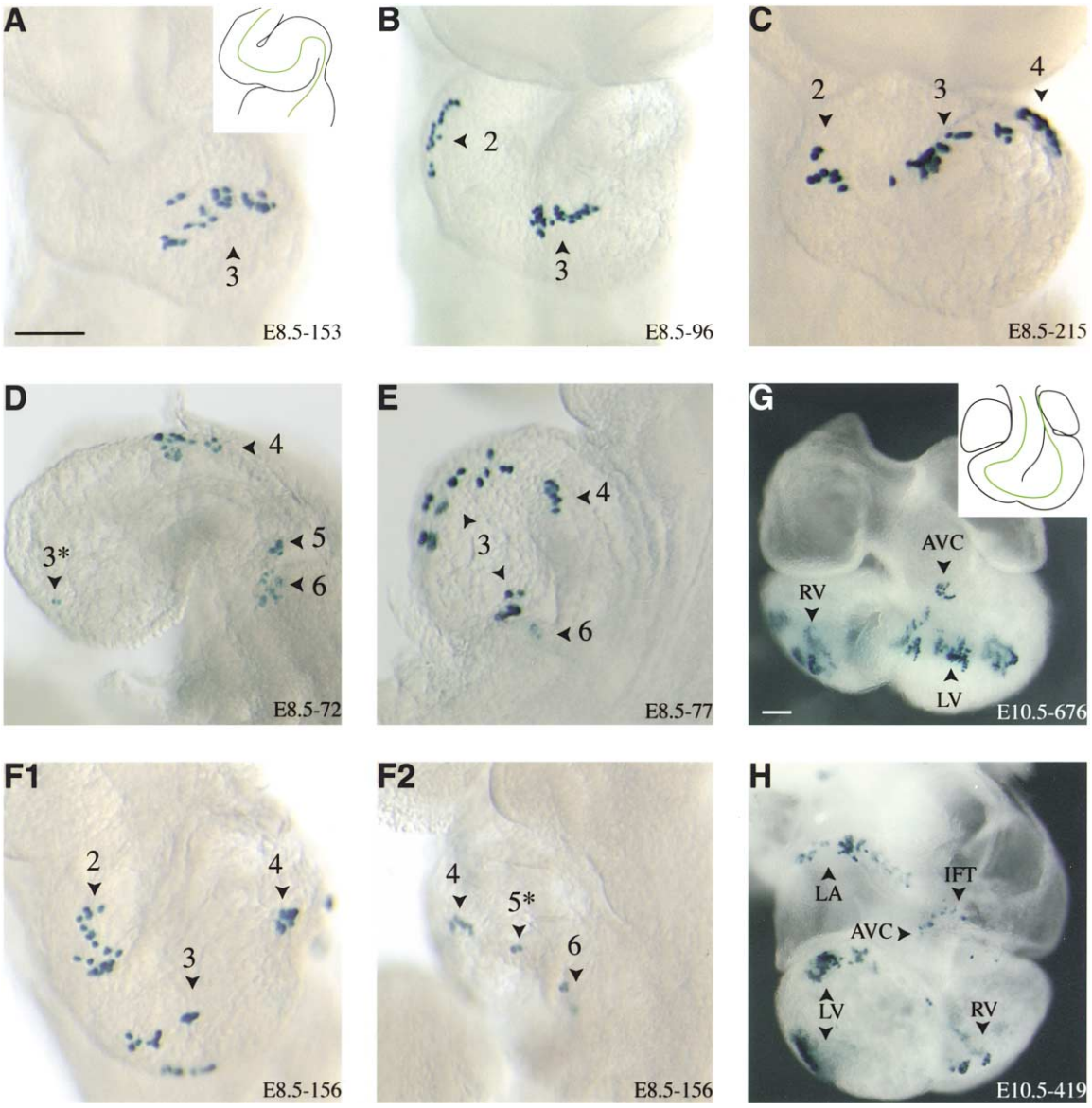

Figure 3. Examples of Clones from the First Lineage

(A–F) Examples of clones at E8.5 colonizing the primitive left ventricle (region 3) are shown, with an increasing extension along the arterial-venous axis of the cardiac tube. Note the participation of larger clones (D–F) in the venous region 6. (A)–(C) and (F1) are ventral and (D), (E) and (F2) left lateral views.

(G and H) Examples of positive hearts at E10.5 with staining in the left ventricle (LV). Note the similar extension along the arterial-venous axis of the distribution of β-galactosidase-positive cells in (G) and (C), and (H) and (F). (G) is a superior view, after removal of the (negative) outflow tract. (H) is an inferior view. AVC, atrio-ventricular canal; IFT, inflow tract; LA, left atrium; RV, right ventricle. The arterial-venous axis is shown in green in the two insets (A and G). Scale bars: 250 μm.

(Figure 5A1), with an equal contribution to region 2 and 6/7 (Figure 5A2) and a predominant contribution of clones from the first lineage to region 4 and 5. Within these regions, which are defined by morphological criteria, we have assessed in more detail the spatial distribution of cells from the two lineages, to determine whether and where a clonal boundary exists. In region 2—presumptive right ventricle—(Figure 5B), both lineages overlap in the ventral part (Figure 5B1), whereas cells of the second lineage are predominant in the dorsal aspect (Figure 5B2). In regions 4 and 5 (Figure 5C), the left part is mainly colonized by cells of the first lineage (Figure 5C1), whereas both lineages colonize the right part (Figure 5C2). If we now distinguish regions 6 and 7, in region 7—primitive right atrium—(Figure 5D), two complementary domains tend to be colonized by each lineage, whereas there is more intermingling of cells from either source in region 6—primitive left atrium. These results indicate that the first and second lineages have overlapping contributions on the arterial-venous

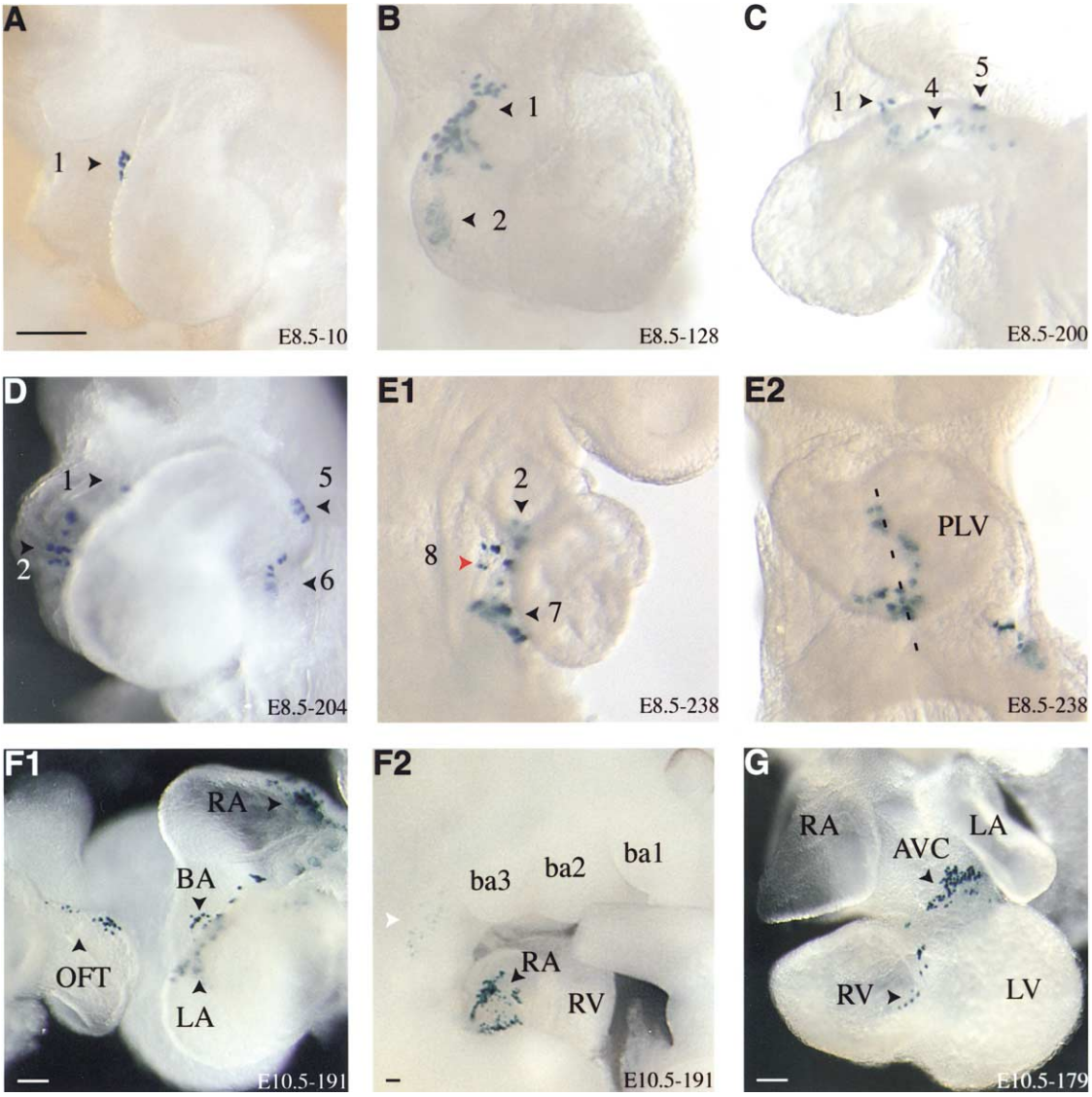

Figure 4. Examples of Clones from the Second Lineage

(A–D) Examples of clones at E8.5 colonizing the arterial region 1 are presented, with an increasing extension along the arterial-venous axis of the cardiac tube. (E) Example of a clone at E8.5 with β-galactosidase-positive cells in the dorsal mesocardium (red arrowhead). The dotted line shows the restriction of labeling to the inner curvature. (B) and (E2) are ventral, (A), (C), and (D) left lateral, and (E1) right lateral views.

(F) Example of a positive heart at E10.5 with staining in the outflow tract and atria but not in the left ventricle. Note the staining in the caudal pharyngeal region (white arrowhead in [F2]).

(G) Example of a positive heart at E10.5, similar to the clone E8.5-177 (Figure 2B), with staining in the inner curvature, continuously from the atrio-ventricular canal (AVC) to the right ventricle (RV). The (negative) outflow tract and part of the right ventricle have been removed for better visualization. (F1) is a dorsal view of the heart, (G) is a superior view, and (F2) is a right lateral view of the embryo. AVC, atrio-ventricular canal; ba, branchial arch; BA, body of the atrium; LA, left atrium; LV, left ventricle; OFT, outflow tract; RA, right atrium; RV, right ventricle. Scale bars: 250 μm.

axis of the cardiac tube, in regions 2, 4, 5, and 7. However, each lineage appears to have a preferential contribution on another axis, relative to the circumference of the cardiac tube.

Common Clonal Origin of First and Second Myocardial Lineages

The category of very large clones (in red and black stripes Figure 2B) may either correspond to a third population of clones or to common precursors of the first and second lineages, as represented schematically in Figure 6E. In addition to the higher number of labeled cells, the very large clones do not have a distinct distribution, but encompass the characteristics of the first and second lineages in their more extensive contribution to regions of the arterial-venous axis. Furthermore, very large clones are the rarest (3 out of 206 positive embryos at the looped heart tube stage), suggesting that they derive from more ancient precursors. Therefore, the subclones of the very large clones, which necessarily

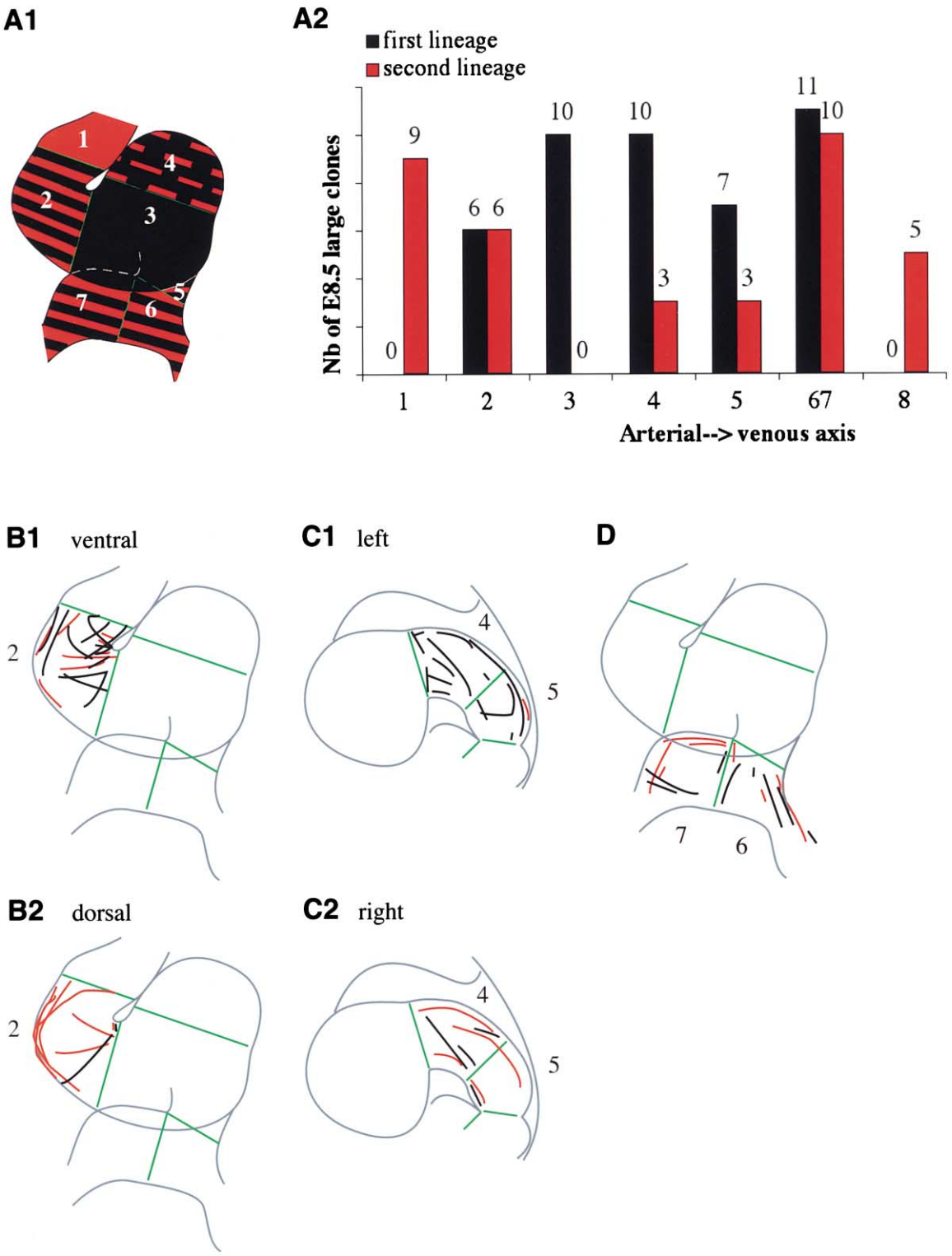

Figure 5. Overlap of the Contributions of the First and Second Lineages to the Cardiac Tube at E8.5

(A1) Summary of the participation of each lineage in different regions of the cardiac tube. (A2) Frequency of large clones (see Figure 2B) observed in each region, from either the first (black) or second (red) lineage.

(B–D) More detailed representation of the distribution of cells from the first (black) or second (red) lineage, in the regions (2 in [B], 4 and 5 in [C], and 6 and 7 in [D]) to which both categories contribute. In this qualitative representation, small clones which can be clearly attributed to a lineage (on the basis of their participation in either region 3 or 1) are included as well as large clones. (B1) represents the ventral contribution and (B2) the dorsal. (C1) represents the left contribution and (C2) the right.

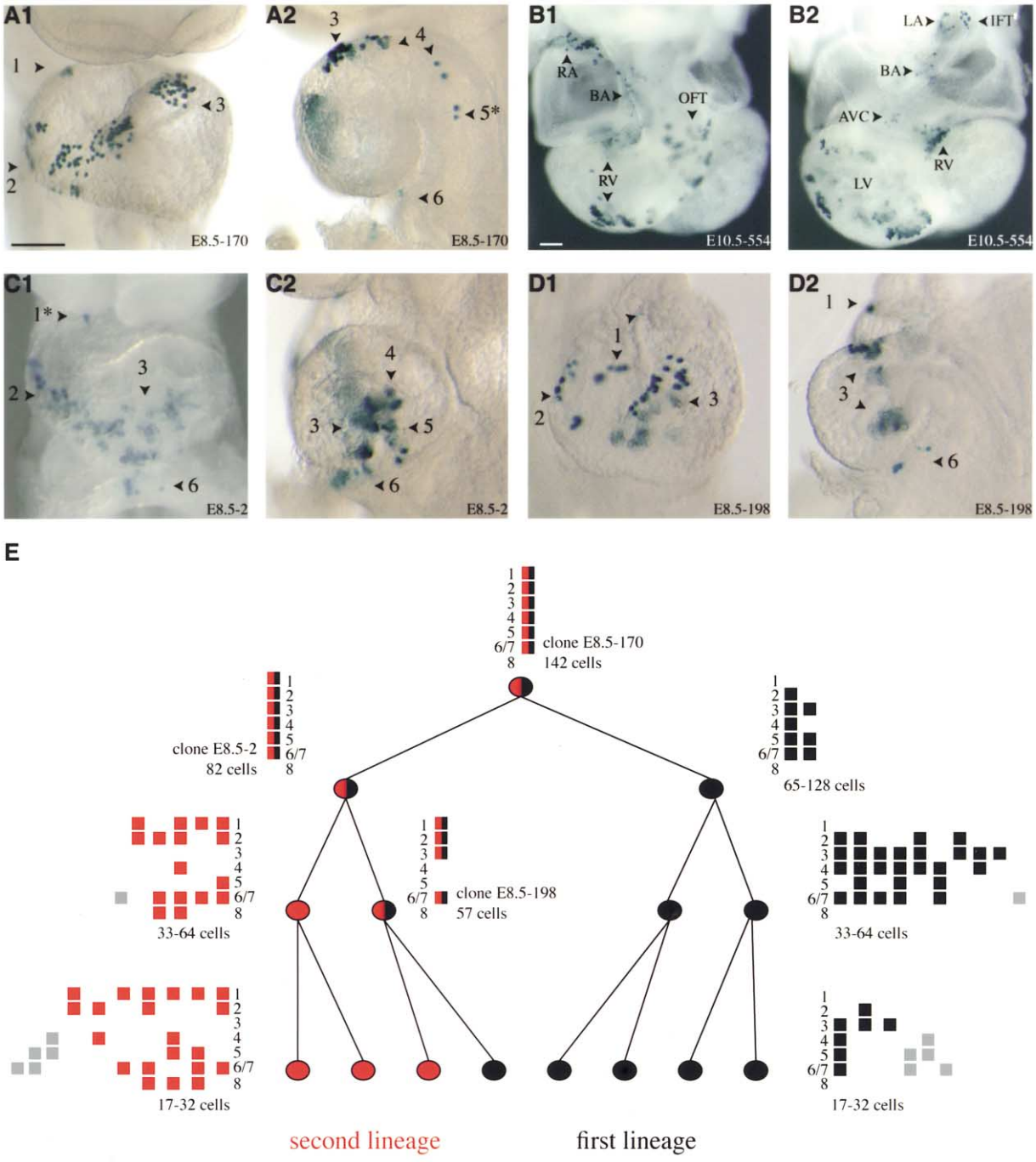

Figure 6. Common Clonal Origin of the First and Second Lineages

(A, C, and D) Photographs of the very large clones at E8.5, which colonize both regions 1 and 3. (A1), (C1), and (D1) are ventral and (A2), (C2), and (D2) left lateral views.

(B) Example of the positive heart at E10.5 containing the highest number of labeled cells (1,000 observed), which are distributed in every region. (B1) is a superior and (B2) an inferior view. AVC, atrio-ventricular canal; BA, body of the atrium; IFT, inflow tract; LA, left atrium; LV, left ventricle; OFT, outflow tract; RA, right atrium; RV, right ventricle. Scale bars: 250 μm.

(E) Lineage tree of myocardial precursor cells, based on the exponential increase of labeled cells in a clone at each division. Circles indicate precursor cells of the first (black) or second (red) lineage or a common precursor of both lineages (black and red). The very large clones which colonize all parts of the cardiac tube are placed at the top of the lineage tree, as precursors of the two lineages. Since clones which correspond to the first lineage are larger than those of the second lineage, the first lineage is represented as emerging earlier than the second. The characteristics of clones (number of cells and participation in the cardiac tube, as schematized in Figure 2) corresponding to the precursor cells at a given stage represented in the lineage tree are indicated on either side. For clarity, clones with redundant patterns are shown once. Thus, in the size class of 33–64 cells, first lineage clones E8.5-102 and E8.5-215 (see Figure 2B) are represented once. Small clones, which cannot be specifically attributed to one lineage, are indicated in gray.

exist, are very probably constituted by at least a portion of large clones from the first or second lineages. First and second lineages therefore appear to share early common precursors.

Number of Myocardial Precursor Cells and Segregation of the Lineages

The number of myocardial precursor cells can be estimated on the basis of the proportion of the tube which is colonized by a clone. The largest clone observed at E8.5 contains 142 cells (Figure 2A2; Figure 6A) distributed throughout the length of the cardiac tube. Considering that the E8.5 cardiac tube contains approximately 19,000 cells (Meilhac et al., 2003), this corresponds to a pool with about 19,000/142≈140 myocardial precursors, which are not restricted in their contribution to the arterial-venous axis of the cardiac tube. Similarly, the heart at E10.5 with the highest number of β-galactosidase-positive cells (Figure 6B) also shows colonization of all regions. It is estimated to have about 1000 labeled cells, which represents about 1/180th of the total number of myocardial cells at this stage (Meilhac et al., 2003). Given the uncertainty in counting the total number of myocardial cells per heart, this is not very different from the figure of 140, estimated at E8.5 for the number of myocardial precursors.

A lineage tree of myocardial precursor cells can be reconstructed (Figure 6E), based on the exponential increase in the number of clonal descendants, as discussed previously (see second paragraph of the Results section). The largest clones from the first lineage have more cells (75, 85, or 91 cells, see Figure 2B) than those from the second (36, 37, 40, or 52 cells, see Figures 2B and Figures 4D and 4E) and reach the number seen for one member of the category of very large clones, which colonizes the whole length of the cardiac tube (E8.5-2 with 82 cells, Figure 6C). Assuming that both lineages have equal proliferation rates, this suggests that the first lineage arises before the second (Figure 6E). A very large clone (E8.5-198 with 57 cells, Figure 6D), classed in this category because it colonizes both regions 1 and 3, is still observable after the appearance of the first and second lineages, but does not extend to all regions of the cardiac tube, as distinct from the other very large clones. Therefore, the age of clones is confirmed by their degree of regionalization. In the size class of 33-64 cells (Figure 6E), corresponding to the largest clones from the second lineage, there are five clones from the second lineage (including four with a large extension ≥3) and ten from the first (including seven with a large extension). This suggests that from the pool of common precursors, about two-thirds will be allocated to the first lineage and one-third to the second lineage.

Individualization of Regions at the Extremities of the Cardiac Tube

Since each myocardial lineage contributes to a large part of the heart, they do not account for the segregation of adjacent regions on the arterial-venous axis, which constitute the presumptive functional units of the heart. As examples, individualization of regions 1, 2, and 6/7 was investigated, focusing on small clones, which are restricted to a single region (Figure 2A1) or contribute to two adjacent regions (Figure 2A2). Clones at E8.5 contributing to two regions are more frequent in the case of regions 1 and 2 and can have as few as seven cells (Figure 7A), suggesting that cells from these two regions can still arise from a common precursor three divisions earlier. As shown in Figure 7B, clones contributing to regions 1 and 2 extend largely over the boundary, indicating that this would be true also if the boundary had been chosen more anteriorly or more posteriorly. Consistently, clones restricted to either region 1 or 2 contain a lower number of cells than clones that cross the boundary (Figure 7A). These observations therefore show that, even three divisions before E8.5, there is no clonal boundary between regions 1 and 2. This situation is in contrast to that at the venous pole of the heart where restricted clones are very frequent in region 6/7, containing as many as 42 cells (Figure 7A). Only three clones extend over the boundary with region 5, including one which barely extends over the boundary. This suggests that region 6/7 is individualized earlier than regions 1 and 2.

At the arterial and venous poles of the heart, labeled cells extend into adjacent vessels. A continuous distribution of β-galactosidase-positive cells is observed in the outflow tract and the aortic sac (five cases at E10.5, Figure 7C). Notably, this is seen also for clones with a low number of cells (≥6 cells), in agreement with a late individualization of region 1. β-galactosidase-positive cells extend from the right atrium to the right superior caval vein (three cases at E10.5, Figure 7D), from the left atrium to the left superior caval vein (four cases at E10.5, Figure 7D), or from the left atrium to the forming pulmonary vein (two cases at E10.5 and one at E14.5, Figure 7E). In these cases, the phenomenon is seen for clones with a higher number of cells (≥75 cells), consistent with the earlier individualization of the atria. These results show that cells, which express α-*cardiac actin* in the great vessels, can share clonal relationships with myocardial cells. This is in agreement with previous studies based on molecular markers (Lyons et al., 1990; Millino et al., 2000) and morphological observations (Webb et al., 1998).

Discussion

The retrospective clonal analysis reported here demonstrates the clonal segregation of two populations of myocardial precursor cells, which constitute two myocardial lineages. They share an early common clonal origin and seem to appear sequentially. The analysis at E8.5 reveals that the second lineage contributes to both poles of the heart and identifies the left ventricle as the only region which is exclusively colonized by the first lineage, whereas the outflow tract is entirely formed from the second lineage. Adjacent regions, which correspond approximately to the future functional units of the heart, do not acquire their regional identity at the same time. The atrial region (6/7) appears to be clonally distinct well before E8.5, whereas the presumptive right ventricular region (2) has not segregated from the outflow region (1) until shortly before E8.5.

A

Regions colonised	Min-Max number of cells	Number of clones
1	3-10	7
2	3-7	4
6/7	4-42	16
1+2	7-54	10
5+6/7	12-30	3

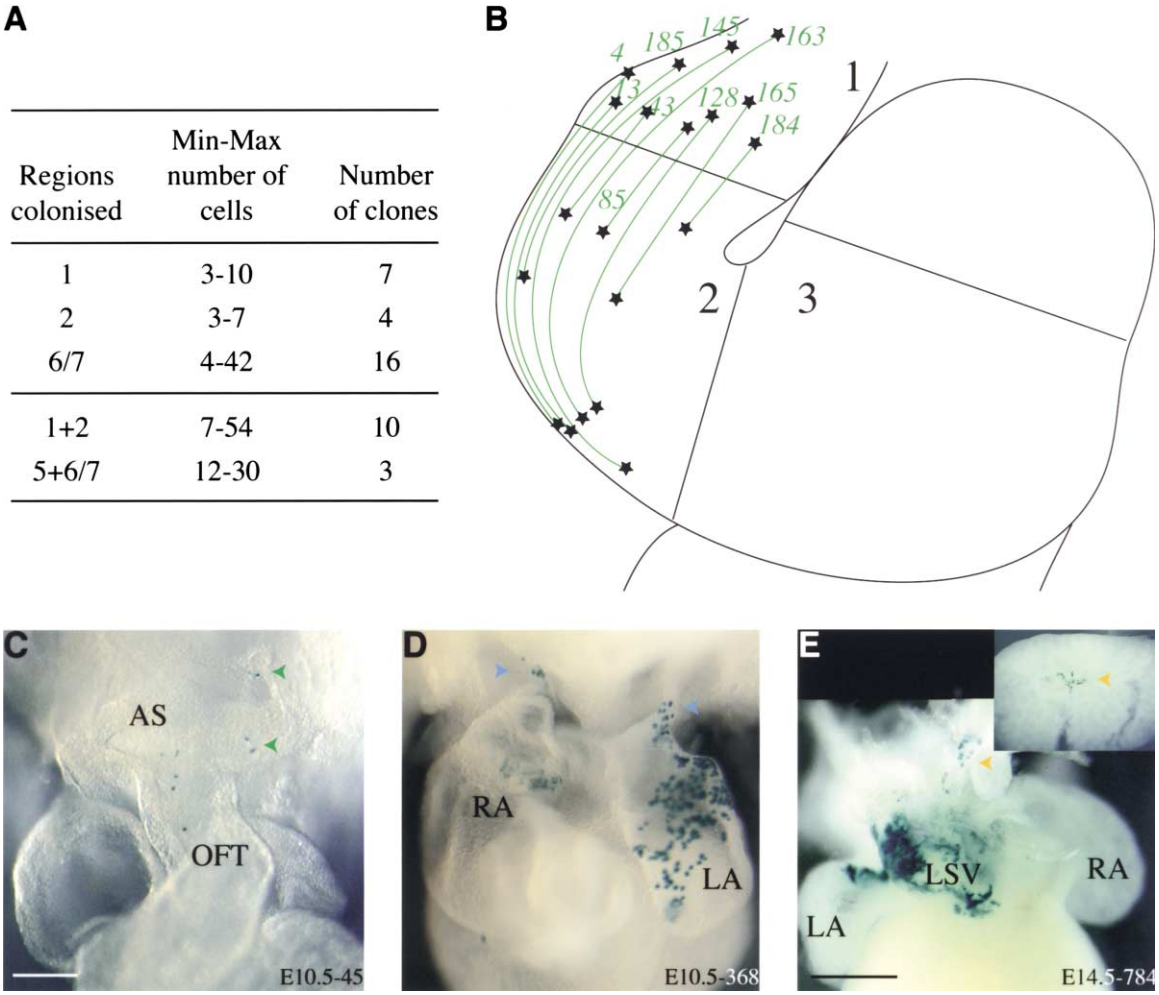

Figure 7. Individualization of Regions at the Extremities of the Cardiac Tube

(A) Table summarizing the properties of small clones in regions 1, 2, and 6/7 (see Figure 2A).

(B) More detailed representation of small clones at E8.5, extending over the boundary between regions 1 and 2. Only the anterior and posterior limits of a clone are taken into account (stars), joined by a green line along the arterial-venous axis. Green numbers identify the clones and black numbers the cardiac regions.

(C–E) Participation of clones of myocardial cells in the walls of the great blood vessels. (C) Example of a positive heart at E10.5 with labeled cells in the outflow tract (OFT) and aortic sac (AS, green arrowheads). (D) Example of a positive heart at E10.5 with labeled cells in the left (LA) and right (RA) atria and in the left and right superior caval veins (blue arrowheads). (E) Example of a clone at E14.5 colonizing the left atrium, the left sinus venosus (LSV), and the pulmonary vein (yellow arrowhead). The inset shows a lobe of the lung from the same embryo, also containing labeled cells. Scale bars: 500 μm.

Characterization of Myocardial Founder Cells

Our results point to the existence of a population of myocardial founder cells, which are not restricted in their participation along the arterial-venous axis of the E8.5 cardiac tube. In the chick, DiI injections at HH5—just after gastrulation—have resulted in second lineage-like patterns, with labeled cells in both extremities of the heart tube at HH12 (Redkar et al., 2001). Earlier labeling of cells in the chick primitive streak led to the suggestion that cardiac precursor cells have a rostro-caudal organization prefiguring that along the arterial-venous axis of the cardiac tube (Garcia-Martinez and Schoenwolf, 1993). In these experiments on chick embryos, the cells labeled do not have the properties of the common precursors, which participate in the whole length of the cardiac tube, described in our analysis. This might suggest that the segregation of the first and second lineages that we observe (Figure 6E) dates back to the onset of gastrulation.

Our quantitative analysis is based on the assumption that myocardial precursor cells grow exponentially (Meilhac et al., 2003) with an overall constant division rate. It provides a first approximation in the mouse of the number of myocardial founder cells, which we estimate to be about 140, and of the relative size of each pool of cells of either lineage (two-thirds/one-third for the first/second lineages). If the earliest precursor labeled here, which gives rise to 142 cells in the E8.5 heart (clone 170), dates back to the onset of gastrulation, at about E6.5, this would suggest that the average rate of proliferation of myocardial precursors is about 7 hr per division. Although only approximate, this is consistent

with other estimations of cell division rates in the E6.5-E7.5 mouse embryo (Lawson et al., 1991) and is much more rapid than that estimated for differentiated myocardial cells (15 hr, Meilhac et al., 2003).

Progressive Regionalization of Myocardial Precursor Cells along the Arterial-Venous Axis

We have not detected any clonal relationship between cells of the future cardiac chambers (approximately regions 2, 3, and 6/7), as distinct from the other regions, which have been described as primary versus working myocardium. This suggests that the initiation of a distinct transcriptional program in cells of the cardiac chambers (Christoffels et al., 2000) is not the consequence of a distinct clonal origin. However, the preferential contribution of cells from the second lineage to the inner curvature as well as their specific contribution to the outflow region, suggest that cells of the second lineage are particularly implicated in the formation of the so-called primary myocardium.

When are myocardial cells being allocated to the future functional regions of the embryonic heart? Our observation that large clones colonize most of the cardiac tube clearly shows that there are no clonally distinct segments during the early stages of cardiogenesis, contrary to a segmental model of heart development (Stalsberg and De Haan, 1969; see Brand, 2003). Previous experiments on chick embryos, which show a tendency toward a rostro-caudal organization of cardiac precursor cells in the chick primitive streak (Garcia-Martinez and Schoenwolf, 1993), do not necessarily imply that labeled cells are restricted to a single region of the cardiac tube, nor that labeling of different precursors does not overlap (Rosenquist, 1970). Our analysis of more recent (small) clones shows that individualization of different regions of the cardiac tube does not occur simultaneously. In particular, the presumptive atria acquire distinct clonal characteristics earlier. This is in agreement with labeling experiments, which have shown the existence of cells with atrial identity at the cardiac crescent stage (Redkar et al., 2001). This is the first stage at which perturbation of the rostro-caudal organization of cardiac precursor cells leads to abnormal heart morphogenesis (Patwardhan et al., 2000), or at which cardiac cells become resistant to variations in retinoic acid signaling (Hochgreb et al., 2003). In contrast to the atria, our results indicate the absence of a clonal boundary between the presumptive right ventricular region and the outflow region, at least until shortly before E8.5.

Given the early dispersive growth of myocardial precursor cells which becomes coherent around E8.5 (Meilhac et al., 2003), embryonic cardiac regions may arise by progressive restriction of cell dispersion, rather than by a process of individualization due to the establishment of a pre-pattern based on lineage specification, which would imply strict clonal segregation of adjacent regions along the arterial-venous axis. This corresponds to the mode of cell allocation in the mouse embryo, as opposed to that in invertebrates (Mathis and Nicolas, 2002). At the molecular level, cardiac transcription factors are often expressed in gradients in the linear heart tube. It is only later that gene expression becomes more clearly restricted and that mutations in genes such as *dHAND* and *Tbx5* affect the formation of particular cardiac chambers (Harvey, 2002). Thus molecular regionalization appears to parallel cellular regionalization. The fact that mutations in these factors cause hypoplasia rather than complete absence of particular cardiac regions is compatible with a mechanism of regionalization which is not strictly defined by lineage. Establishment of strict clonal boundaries may be a later event, which will then influence the distribution of cells in a clone. It is only at the time of the formation of the interventricular septum, around E10.5, that a clonal boundary appears to be set up between the right and left ventricles (Meilhac et al., 2004; S. Zaffran et al., unpublished data). In other tissues of the mouse embryo, such as the rhombencephalon (Mathis et al., 1999) or the myotome (Eloy-Trinquet and Nicolas, 2002), it has also been observed that clonal boundaries between spatial domains are late events. This points to the role of clonal boundaries in mammals in maintaining regionalization, rather than in initiating it, in contrast to the situation in insects (Morata and Lawrence, 1977).

The Relation between the First/Second Lineages and the Primary/Anterior Heart Fields

The two myocardial lineages, distinguished by our clonal analysis, are referred to as first and second lineages because of their participation in the early (region 3) or late (region 1) parts of the cardiac tube. This may be also related to the timing of their segregation, as shown in Figure 6E. These lineages share characteristics with the primary and anterior heart fields which have been described previously in the mouse (Kelly et al., 2001). The primary heart field, represented already in the cardiac crescent, contributes, like the first lineage, to the embryonic left ventricle and the venous pole. In contrast, the anterior heart field contributes cells to the anterior part of the cardiac tube, to the outflow tract, and also to the right ventricle, but not to the primitive left ventricle, which represents most of the early linear heart tube (S. Zaffran et al., unpublished data). The second lineage has similar characteristics. Markers of the anterior heart field at the cardiac crescent stage are localized medially. This is consistent with the observations reported here that clones from the second lineage colonize medial structures, such as the inner curvature of the heart tube as well as the dorsal mesocardium. In addition, it has been shown that, later, cells of the anterior heart field are derived from pharyngeal mesoderm, and this is consistent with the observation of labeled cells from the second lineage in the branchial arches.

However, we show that the second lineage has a wider fate than that previously described for the anterior heart field. Notably, the second lineage can participate not only in the arterial but also in the venous pole of the cardiac tube. Very recently, *Isl-1* has been shown to mark a similar population of myocardial precursor cells (Cai et al., 2003). The degree of overlap of the two heart fields has not yet been investigated. We show here that the first and second lineages have overlapping potentials to contribute to the formation of region 2—presumptive right ventricle, 4 and 5—presumptive atrio-ventricular canal and body of the atrium, and 6 and 7—presumptive atria. The first and second lineages have distinct properties, as shown by the preferential contribution of the

second lineage to the dorsal portion of region 2 and the anterior right portion of region 7—presumptive right atrium, or by the preferential contribution of the first lineage to the left aspect of regions 4 and 5—presumptive atrioventricular canal and body of the atrium. Thus the heart fields, as previously described, do not strictly correspond to the first and second myocardial lineages. This discrepancy probably reflects the techniques used to detect different cell populations. For example, Fgf transgenic markers of the anterior heart field (Kelly et al., 2001; R. Abu-Issa and E. Meyers, personal communication) do not label the venous pole of the heart. This may indicate that the derivatives of Fgf8/10-expressing cells are a subpopulation of the second lineage. Conceptually, the heart fields are based on spatial considerations. DiI labeling, used to define the fields, puts more emphasis on the apparent movement of cells and on their specific incorporation at the anterior or posterior extremity of the cardiac tube, rather than on their clonal origin.

Evolutionary Implications

As the pulmonary circulation emerges during evolution, the vertebrate heart is transformed from a structure with two to a structure with four chambers, when one ventricle and one atrium are added and the outflow tract is developed. In the context of the segmented model of heart morphogenesis, evolution of the vertebrate heart was proposed to result from the addition of genetic modules (Fishman and Olson, 1997). In this context, the anterior heart field may correspond to a later evolutionary addition. However, we show here that the second lineage also contributes to both atria and therefore does not only correspond to the regions added in the course of evolution. Furthermore, the right ventricle also has a contribution from the first lineage. It has been shown in the zebrafish, which has a single atrium and ventricle, that there is an early segregation of ventricular and atrial cells, before gastrulation (Stainier et al., 1993). Two distinct lineages thus appear to exist also in the zebrafish and to segregate early. An alternative evolutionary hypothesis is that the last common ancestor of fish and mammals already had two myocardial lineages, which have then diversified in mammals, so that they no longer correspond strictly to atrial versus ventricular precursors. As an evolutionary intermediate between zebrafish and mouse, the chick heart has also been shown to be derived from two heart fields (Waldo et al., 2001; Mjaatvedt et al., 2001), but the second appears to contribute only to the outflow tract and not to the right ventricular region, in contrast to the situation in the mouse. In this context, it will be of major interest to investigate the clonal segregation of myocardial cells in an amphibian such as *Xenopus*. A progression by cooption for myocardial cell lineages would be consistent with a general tendency observed at the cellular (Mathis and Nicolas, 2002) as well as the molecular (Erwin and Davidson, 2002) level during invertebrate and vertebrate evolution.

Experimental Procedures

Production of Clones

Random clones of β-galactosidase-positive cells were spontaneously generated in embryos from the α-*cardiac actin*$^{nlaacZ1.1/+}$ line, as previously described (Meilhac et al., 2003, 2004). A total of 3629 α-*cardiac actin*$^{nlaacZ1.1/+}$ embryos were dissected at E8.5, including 238 with β-galactosidase-positive cells in the myocardium. For homogeneity, only positive embryos (206) at the looped cardiac tube stage (9–17 somites) were taken into account here. Examples of positive hearts at E10.5 (Figures 3G–3H, 4F–4G, 6B, 7C–7D) and E14.5 (Figure 7E) are provided, to show the distribution of β-galactosidase-positive cells with respect to more mature cardiac morphology.

In addition to the large number of clones examined in this analysis, the observation of similar clones several times (see for example clones E8.5-156, E8.5-237, and E8.5-25 in Figure 2A2) indicates that we are probably close to saturation in detecting the different categories of clones.

Clonal Relationship between β-Galactosidase-Positive Cells at E8.5

The intragenic recombination, which converts *nlaacZ* into *nlacZ* is a spontaneous, heritable, and random event. The frequency of its occurrence can therefore be analyzed by the fluctuation test of Luria and Delbrück (1943): the rate of recombination of *nlaacZ1.1* in the α-*cardiac actin* locus and in the E8.5 heart, which contains about 19,000 cells (Meilhac et al., 2003), is 3.6×10^{-6} per cell and per division, given the number of negative embryos. The fluctuation test of Luria and Delbrück also predicts that the number of independent recombinations follows a Poisson distribution. Based on the number of observations here (see above), we can calculate that eight cases of two independent recombination events might be expected and none with more. Since hearts with a low number of β-galactosidase-positive cells are more frequent—54% contain one or two cells—the examples in which two independent recombination events have occurred may well correspond to the hearts in which one to two labeled cells are isolated from the others (see E8.5-199, E8.5-122, E8.5-91, E8.5-79, and E8.5-4 in Figure 2A). Extreme examples are provided by the three hearts containing three labeled cells, with, respectively, one and two cells in distinct regions, and are not represented in Figure 2. Positive regions containing one to two labeled cells are represented by asterisks (Figures 2, 3, and 6) in the 17 clones concerned. A second recombination event may occur in any positive heart. However, since small clones (Figure 2) are more frequent (87%), secondary recombination events will occur more frequently in this category of clones. Regions containing one to two labeled cells were therefore not taken into account for the calculation of the extension of the 11 small clones (Figure 2). Furthermore, to do so would have created nonsignificant categories of clones, such as that of E8.5-122, which has no counterpart. In the case of the large clones (Figure 2B), clusters of one to two labeled cells were retained in the analysis, because their distribution pattern is consistent with that of other clones.

Statistical Analysis

The frequency of two independent events is equal to the product of the probability of each single event. Thus the calculated number of observations of both independent events A and B (Table 1) is $C = N_A * N_B / N_t$ where N_A is the number of observations of event A, N_B of event B, and N_t the total number, i.e., 3629 α-*cardiac actin*$^{nlaacZ1.1/+}$ embryos dissected at E8.5.

Conformity of a frequency to a theoretical law was assessed by the classical χ^2 test, calculating the parameter $\chi^2 = (O\text{-}C)^2/C$, where O is the observed frequency and C the calculated frequency (1 degree of freedom).

Schematic Representations of the Clones at E8.5

Clones were described in terms of their participation in each region, and this information was classified using Microsoft Excel software (Figure 2). The participation of clones in a given region was described spatially using Adobe Illustrator (Figures 5A1, 5B–5D, and 7B).

Histological Sections

Embryos, which were fixed in 4% paraformaldehyde and stained with X-gal, were embedded in 15% sucrose/7% gelatin in PBS,

and sectioned on a cryostat. The procedures were as previously described (Teboul et al., 2002).

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