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Oriented clonal cell growth in the developing mouse myocardium underlies cardiac morphogenesis

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During heart morphogenesis, cardiac chambers arise by differential expansion of regions of the primitive cardiac tube. This process is under the control of specific transcription factors such as Tbx5 and dHAND. To gain insight into the cellular mechanisms that underlie cardiogenesis, we have used a retrospective clonal approach based on the spontaneous recombination of an *nlaacZ* reporter gene targeted to the murine *α-cardiac actin* locus. We show that clonal growth of myocardial cells is oriented. At embryonic day (E) 10.5, the shape of clones is characteristic of a given cardiac region and reflects its morphology. This is already detectable in the primitive cardiac tube at E8.5, and is maintained after septation at E14.5 with additional modulations. The clonal analysis reveals new subdivisions of the myocardium, including an interventricular boundary region. Our results show that the myocardium, from the time of its formation, is a polarized and regionalized tissue and point to the role of oriented clonal cell growth in cardiac chamber morphogenesis.

Introduction

The cellular processes by which organs acquire their structure are poorly understood. The heart, which is the first organ to form in the embryo, provides a striking example of morphogenesis. Heart morphogenesis in avian and mammalian embryos involves the formation of a tube from which cardiac chambers expand. The overall structure is deformed by a complex process of torsion and looping (Harvey, 2002). As development proceeds, regions of the myocardium become distinguishable along the arterial–venous axis of the cardiac tube. They correspond to future functional units and are organized in series: the outflow tract, the right ventricle, the left ventricle, the atrioventricular canal, the left atrium, the right atrium, and the inflow tract. These regions may be separated by septum or by valves, and acquire specific connections during the fetal period to form the adult four-chambered heart with two blood flows. The origin and timing of addition of these regions to the cardiac tube is still under investigation. They may be added progressively as distinct segments of the cardiac tube (de la Cruz et al., 1989), or the cardiac chambers may form by ballooning of the so-called working myocardium at the outer curvature of the primitive tube (Christoffels et al., 2000a). In addition, two cell populations or heart fields have been identified that contribute to the myocardium (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). During development, cardiac regions can be distinguished on the basis of their expression of endogenous genes (Franco et al., 1998) or transgenes (Kelly et al., 1999), further insight being provided by expression patterns on mutant backgrounds in which morphology and function are altered.

Key molecules that play a role in shaping the heart tube have been identified as a result of gene manipulation in the mouse. The zinc-finger transcription factor GATA4 is required in the endoderm for early formation of the cardiac tube (Harvey, 2002). Other factors, such as the transcription factors MEF2C, Nkx2.5, dHAND, Tbx5, or Raldh2, involved in retinoic acid signaling, are necessary for the completion of cardiac looping and the expansion of particular cardiac chambers. However, how these molecules affect cell behavior remains poorly understood. Cell proliferation may well intervene. Indeed, Tbx5 (Hatcher et al., 2001), HOP, a recently discovered downstream effector of Nkx2.5 (F. Chen

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Abbreviations used in this paper: α_c*-actin*, *α-cardiac actin*; E, embryonic day.

et al., 2002; Shin et al., 2002), and retinoic acid signaling (T.H. Chen et al., 2002) have been shown to be directly involved in cardiomyocyte proliferation. Regional differences in growth rates have been described in the looped cardiac tube of the chicken, with less proliferation in the inner curvature, the atrioventricular canal, and the outflow tract (Thompson et al., 1990). Thus, differential cell growth is probably involved in heart looping (Stalsberg, 1969) and ventricular wall thickening (Mikawa et al., 1992), but is not sufficient to account for cardiac chamber expansion. The implication of another aspect of cell growth in heart morphogenesis, namely its orientation, has not been investigated. Recently, it has been shown that shaping of other systems, such as the *Drosophila* wing (Resino et al., 2002) or the *Antirrhinum* petal (Rolland-Lagan et al., 2003), largely depends on oriented cell growth rather than on regional differences in growth rates. However, such a mechanism has not yet been examined in the context of mammalian organogenesis.

To examine the existence and role of oriented cell growth during mouse heart morphogenesis, we have adopted a clonal analysis approach based on the use of a modified *lacZ* gene (*nlaacZ*), which is spontaneously activated as a result of a rare intragenic recombination event at a low frequency (Bonnerot and Nicolas, 1993). An *nlaacZ* reporter gene has been integrated into the *α-cardiac actin* (α_c*-actin*) locus (Meilhac et al., 2003), allowing retrospective tracing of β-galactosidase–positive myocardial cells and their precursors. Previously, we have shown that these cells follow a proliferative mode of growth, with an initial dispersive phase oriented along the arterial–venous axis of the cardiac tube, followed by coherent cell growth. It is this later phase, initiated by embryonic day (E) 8.5, which is considered here. Clusters of clonally related cells are anisotropic; they have an elongated shape with a further level of organization into secondary rows of cells. In the present article, we document the form of clusters of β-galactosidase–positive cells in relation to heart morphology at three developmental stages (E8.5, E10.5, and E14.5). We show that myocardial cell growth is oriented at E10.5, and that clones in each region of the heart present distinct and characteristic patterns. Computer simulation indicates that orientation of division as a factor is, by itself, sufficient to generate such shapes of cell clusters. The analysis further reveals cellular subdivisions of the myocardium, such as a distinct region at the interventricular boundary. Oriented clonal cell growth, which is already initiated in the early cardiac tube and persists at fetal stages, underlies the shaping of the heart, suggesting that it is a key parameter of cardiac morphogenesis.

Results

Clonal analysis of β-galactosidase–positive cells was mainly performed at E10.5, when the different cardiac chambers can be identified morphologically in a heart tube that has not yet undergone septation and extensive remodelling, which involves apoptosis (Fisher et al., 2000). 1,155 α_c-actin$^{+/nlaacZ1.1}$ embryos have been analyzed at this stage, including 734 with β-galactosidase–positive myocardial cells. Table I summarizes the number and characteristics of the observations. The orientation of clonal growth was described in terms of the main axis of elongation of β-galactosidase–positive cell clusters, which are clonally related (see Materials and methods). To assess the dynamics of the orientation process, β-galactosidase–positive clones were also examined earlier in the E8.5 heart tube (mainly at the 9–17-somite stage, when looping is complete), and later in the E14.5 heart, when septation is complete and remodeling is underway. Cardiac subregions were analyzed individually, based on their morphology: outflow tract, right ventricle, interventricular region, left ventricle, atrioventricular canal, body of the atrium, and right and left atrial appendages.

Table I. **Number of observations**

Cardiac subregions	Number of E10.5 hearts with *n* βgal-positive cells in a subregion	
	$5 \leq n \leq 40$	$n \geq 40$
Outflow tract	19	7
Right ventricle	32	8
Interventricular region	21	4
Left ventricle	39	11
Atrioventricular canal	9	3
Body of the atrium	6	4
Right atrium	8	7
Left atrium	10	3

Out of 1,155 α_c-*actin*$^{+/nlaacZ1.1}$ embryos dissected at E10.5. βgal, β-galactosidase.

Outflow tract

In the distal part of the outflow tract at E10.5, β-galactosidase–positive cells that are clonally related (see Materials and methods; Meilhac et al., 2003) are distributed along the arterial–venous axis of the cardiac tube (Fig. 1, A, B, F, and G; black arrowheads). These columns have the width of a few cells superiorly on the superior/inferior axis (Fig. 1, A and B). In contrast, cells are more scattered inferiorly (Fig. 1, F and G) and can cover the entire half-circumference (Fig. 1 G). In the proximal part of the outflow tract, clonally related cells are distributed in coherent rows with a circumferential orientation, both superiorly (Fig. 1, B and C; white arrowheads) and inferiorly (Fig. 1, G and H; white arrowheads). Similar orientations are found in large as well as small clones at E10.5 (Fig. 1, compare D with I and E with J), which arise from early as well as recent precursors, respectively (see Materials and methods), suggesting that orientation is maintained during embryonic stages.

At E8.5, the outflow tract is not yet fully formed and cells are being continuously added to the arterial pole of the heart from the anterior heart field (Kelly et al., 2001). At this stage, in the anterior extremity of the looping cardiac tube, clones of cells distribute along the arterial–venous axis (eight cases; Fig. 1, K–M). Inferiorly, this distribution of labeled cells also tends to be more scattered (Fig. 1 L), as seen at E10.5 (Fig. 1 G).

Between E10.5 and E14.5, the outflow tract is extensively remodeled (Watanabe et al., 1998). The distal outflow tract loses its myocardial phenotype (Ya et al., 1998) and gives rise to the great arteries in which cells no longer express α_c*-actin*,

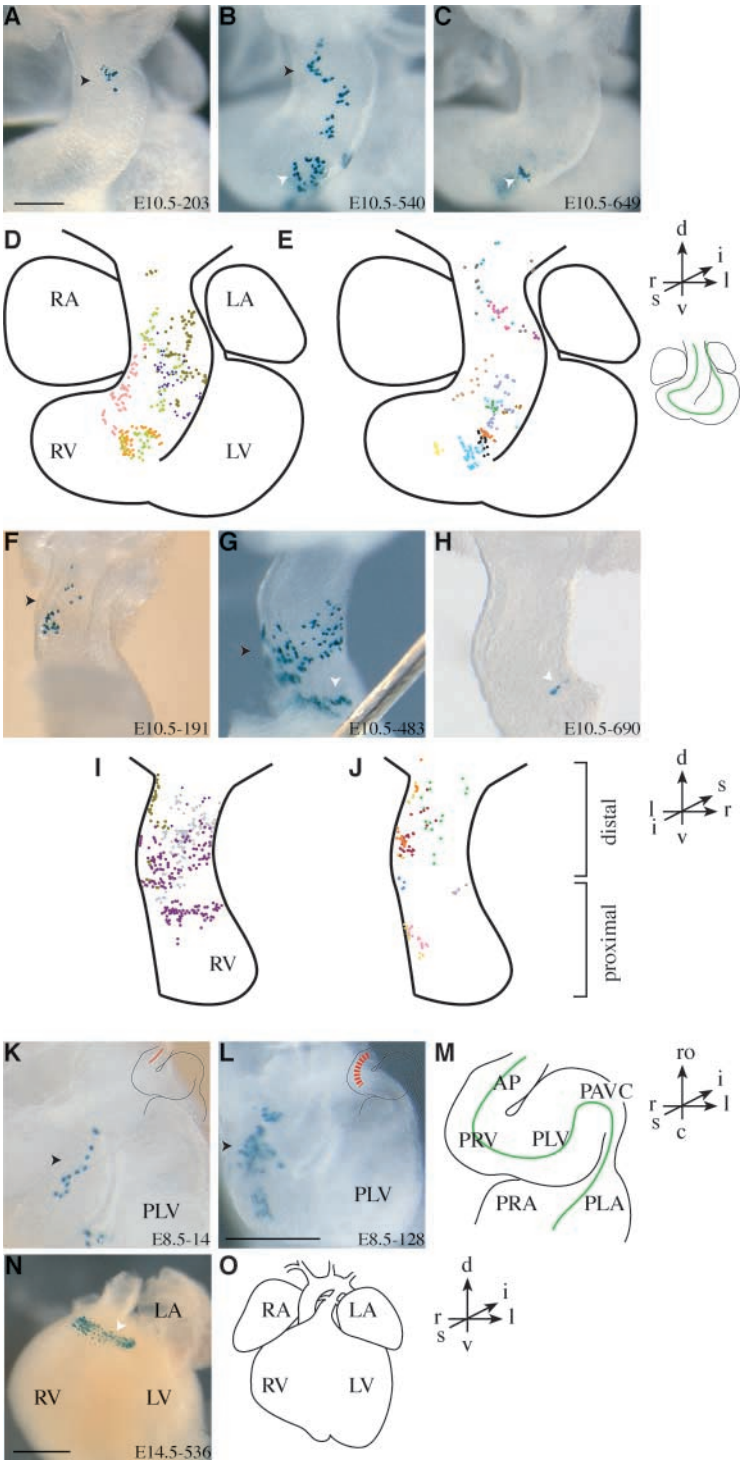

Figure 1. **Clusters of β-galactosidase–positive cells in the outflow tract.** Examples of staining in the outflow tract, viewed superiorly (A–C) and inferiorly (F–H) at E10.5, with a low (A, C, F, and H) or high (B and G) number of β-galactosidase–positive cells. Schematic representations of an E10.5 heart in a superior view (D and E) and of an E10.5 outflow tract in an inferior view (I and J), showing the large ($n \geq 40$ cells in D and I) and small ($5 \leq n \leq 40$ in E and J) clones that were observed in the outflow tract, each in a distinct color. The arterial–venous axis is indicated in green opposite E, and the potential limit between proximal and distal outflow tract opposite J. Examples of large clones in the anterior extremity of the looping cardiac tube at E8.5, in the superior (K) and inferior (L) aspects of the tube (superior views, with labeled cells in L visualized through the tissue). The orientation of clones (in red) is schematized in the insets. (M) Schematic representation of an E8.5 looped heart tube from a ventral view, showing the presumptive regions where clones with distinct properties are detected. The arterial–venous axis is represented in green. (N) An example of a large clone in the outlet of the right ventricle at E14.5 (superior view). (O) Schematic representation of an E14.5 heart from a superior view. Arrowheads indicate a preferential orientation of β-galactosidase–positive cells, which is parallel (black) or perpendicular (white) to the arterial–venous axis. In Figs. 1–6, the numbers in the bottom right corner of the panels indicate the stage followed by the identification number of the positive embryos. AP, arterial pole; AVC, atrioventricular canal; ic, inner curvature; LA, left atrium; LV, left ventricle; oc, outer curvature; OFT, outflow tract; PAVC, presumptive atrioventricular canal; PLA, primitive left atrium; PLV, primitive left ventricle; PRA, primitive right atrium; PRV, presumptive right ventricle; RA, right atrium; RV, right ventricle. The nomenclature superior/inferior is used rather than ventral/dorsal or cranial/caudal, which is confusing because of the looping of the cardiac tube relative to the embryo (see Materials and methods). c, caudal; d, dorsal; i, inferior; l, left; r, right; ro, rostral; s, superior; v, ventral. Bars, 500 μm here and in all other figures.

the locus into which the clonal reporter gene used here was targeted (Meilhac et al., 2003). Most of the proximal outflow tract becomes integrated into the right ventricle in the outlet region (Franco et al., 1997). In this region at E14.5, clonally related cells are organized into circumferential lines (Fig. 1, N and O) of 1–15 cells in width, extending along the entire half-circumference. This is reminiscent of the oriented clonal cell growth observed in the proximal outflow tract at E10.5, which now shows increasing extension on the circumference in a superior view (Fig. 1, white arrowheads; compare N with B).

These results suggest that oriented clonal growth in the presumptive outflow tract at E8.5 underlies the elongation of this portion of the cardiac tube until E10.5. Notably, the columns of cells tend to be twisted in relation to the edge of the outflow tract (Fig. 1 B), which is consistent with a counterclockwise rotation of the heart tube in this region (viewed ventrally; Yelbuz et al., 2002). In the proximal outflow tract at E10.5, the orientation of clonal cell growth is shifted and reflects the enlargement of the cardiac tube until E14.5.

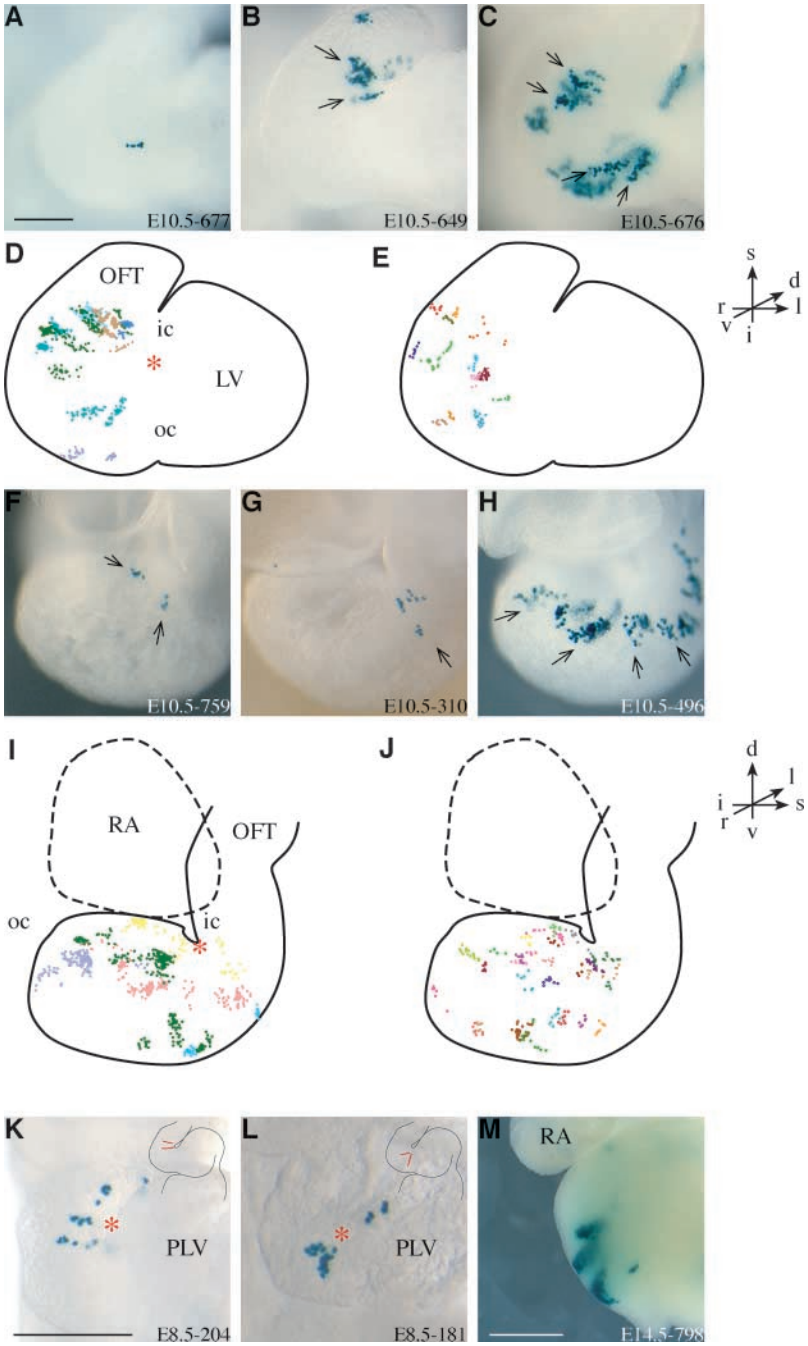

Figure 2. **Clusters of β-galactosidase–positive cells in the right ventricle.** Examples of staining in the embryonic right ventricle at E10.5, in ventral (A–C) and right lateral (F–H) views, with a low (A, F, and G) or high (B, C, and H) number of cells. Arrows indicate the circumferential orientation of β-galactosidase–positive clusters. Schematic representations of an E10.5 heart in the same views (D and E; I and J, respectively), showing the large (D and I) and small (E and J) clones that were observed in the right ventricle, each in a distinct color. Asterisks indicate the points of convergence of the oriented clusters. Examples of large clones in the superior part of the presumptive right ventricular region of the cardiac tube, adjacent to the primitive left ventricle, at E8.5 (K and L, superior views). The orientation of clones (in red) is schematized in the inset. (M) An example of a large clone in the right ventricle at E14.5 (superior view).

Right ventricle

In the E10.5 heart, the embryonic right ventricle is continuous with the outflow tract, but differs in that it has a trabeculated wall. Labeled cells in the embryonic right ventricle are distributed in elongated clusters (Fig. 2, A–C and F–H) that appear circumferential. They radiate from the inner curvature of this portion of the cardiac tube, which is bent back on itself like a hairpin. Notably, the point of convergence is distinct in the ventral (Fig. 2 D, asterisk) and dorsal (Fig. 2 I, asterisk) parts.

At earlier stages, the limitation between the outflow tract and the right ventricle is ill defined. In the arterial extremity of the cardiac tube at E8.5, adjacent to the primitive left ventricle and in a superior view, clonally related cells are also organized in rows with a circumferential orientation radiating from the inner curvature of the cardiac tube (eight cases; Fig. 2, K and L). However, such rows contain only a few cells (Fig. 2, compare K with C), suggesting that the orientation of clonal growth is a recent event. Orientation is along the axis in an inferior view. At E14.5 in the right ventricle, with the exception of the outlet region (Fig. 1 N), larger elongated clusters of β-galactosidase–positive cells are observed with a radial orientation (Fig. 2 M).

Thus, orientation of clonal cell growth in the right ventricle appears to be initiated in the early cardiac tube and corresponds to its enlargement. It is in continuity with the proximal outflow tract from E10.5 onwards. The orientation of clusters in small clones at E10.5 (Fig. 2, E and J), i.e., derived from a recent precursor, is less stereotyped and not always similar to that in large clones (Fig. 2, D and I). This

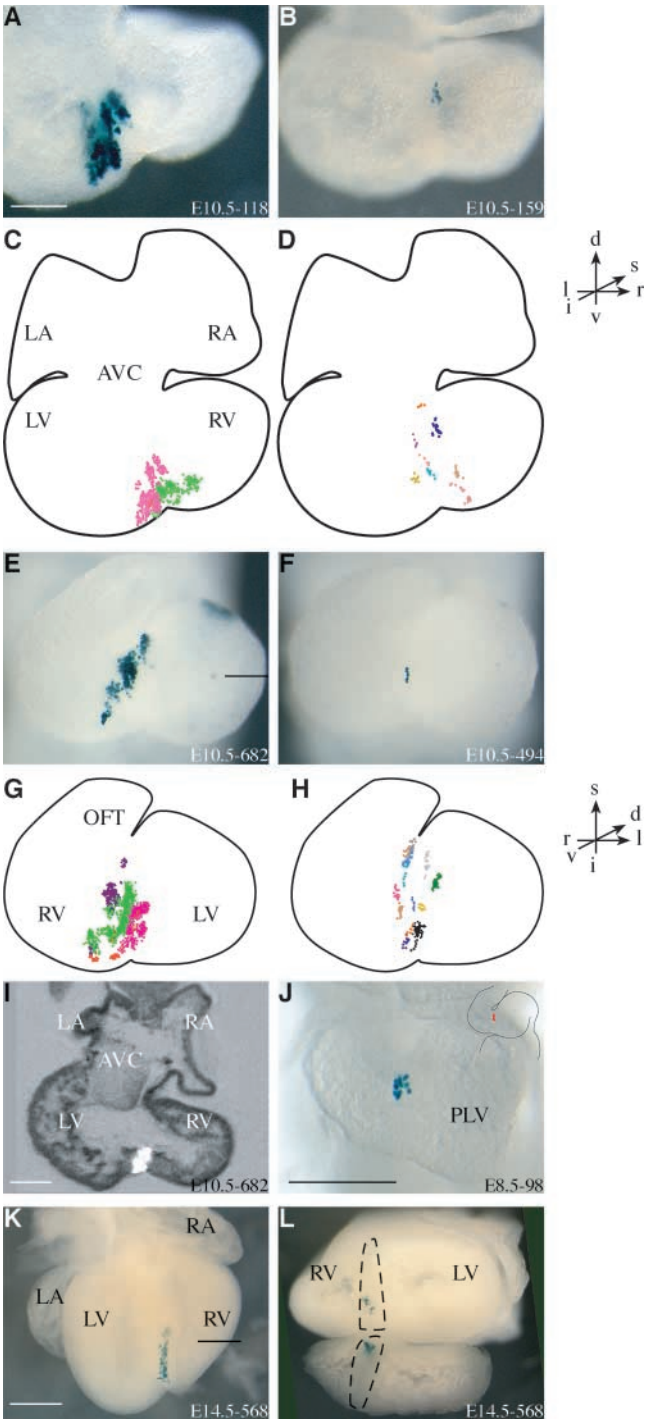

Figure 3. **Clusters of β-galactosidase–positive cells in the interventricular region.** Examples of staining in the interventricular region at E10.5 in inferior (A and B) and ventral (E and F) views, with a high (A and E) or low (B and F) number of cells. Schematic representations of an E10.5 heart in the same views (C and D; G and H, respectively), showing the large (C and G) and small (D and H) clones that were observed in the interventricular region, each in a distinct color. (I) Transverse section of the heart in E, visualized by optical projection tomography at the level indicated by a black bar, showing labeled cells (in white) in the inner primordium of the interventricular septum. (J) An example of a clone at the limit of the primitive left ventricle at E8.5 (superior view). The orientation of the clone (in red) is schematized in the inset. (K) An example of a clone at the level of the interventricular septum at E14.5 (inferior view). (L) Both sides of the transversally sectioned heart in K at the level indicated by a black bar, showing labeled cells in the interventricular septum (within dotted lines).

suggests that a secondary orientation event has begun before E10.5 and is consistent with the suborganization of clusters into secondary rows of cells that prefigure the myofiber architecture of the heart (Meilhac et al., 2003).

Interventricular region

At E10.5, the right and left embryonic ventricles are separated by a shallow groove that marks the primordium of the interventricular septum, extending inwards from the outer curvature. Based on the orientation of clonal growth, the interventricular region emerges as a distinct part of the heart tube. Clusters from large (Fig. 3, A, C, E, and G) and small (Fig. 3, B, D, F, and H) clones are elongated circumferentially in relation to the heart tube along the interventricular groove, showing continuity inferiorly (Fig. 3, A–D) and ventrally (Fig. 3, E–H). Internally, they participate in the primordium of the interventricular septum (transverse section of the heart in Fig. 3 E visualized by optical projection tomography in Fig. 3 I), but are not restricted to it, extending into the adjacent right or left embryonic ventricle (Fig. 3, C and G).

At E8.5, at the limit of the primitive left ventricle, small coherent clusters with a circumferential orientation (three cases; Fig. 3 J) are also observed. They are less elongated than E10.5 clones (Fig. 3, compare J with E), suggesting lower constraints at this early stage. At E14.5, clusters of labeled cells are also found in the interventricular region, where they follow the line of the interventricular septum (Fig. 3 K) and penetrate into it (Fig. 3 L, transversally sectioned heart).

Thus, orientation of clonal growth in the interventricular region has specific properties that underlie the formation of the septum. It may be initiated as early as E8.5 and is still operational at fetal stages. E10.5 clones contain up to 250 cells (Fig. 3 A), which is as many as those in the right (Fig. 2 C) or left (Fig. 4 F) ventricles, but they are organized in highly coherent clusters, occupying a much smaller area. This indicates that the interventricular region has an oligoclonal origin (i.e., derives from a few precursors) and is consistent with previous labeling experiments in the chick, showing that the contribution of ventricular cells to the interventricular septum is limited to a narrow region width of 400–500 μm, centered on the septum (Harh and Paul, 1975).

Left ventricle

In the E10.5 heart, the embryonic left ventricle appears as a bulge on the outer curvature of the cardiac tube. In this region, clonally related cells are distributed into clusters displaying three preferential orientations (Fig. 4, A–J). In the superior view, labeled cells align along the limit of the bulge on the inner curvature (Fig. 4, A and B, gray arrowheads; see also Fig. 4 D). At the external edge of the bulge, clusters follow the outer curvature (Fig. 4 F, black arrowhead; see also Fig. 4, D and I). Elsewhere, clusters have a perpendicular orientation to the inner limit of the bulge, both superiorly (Fig. 4, B and C; white arrowheads) and inferiorly (Fig. 4 G, white arrowhead).

At E8.5, the primitive left ventricle has already begun to bulge out from the linear heart tube (Fig. 4 K). Similar ori-

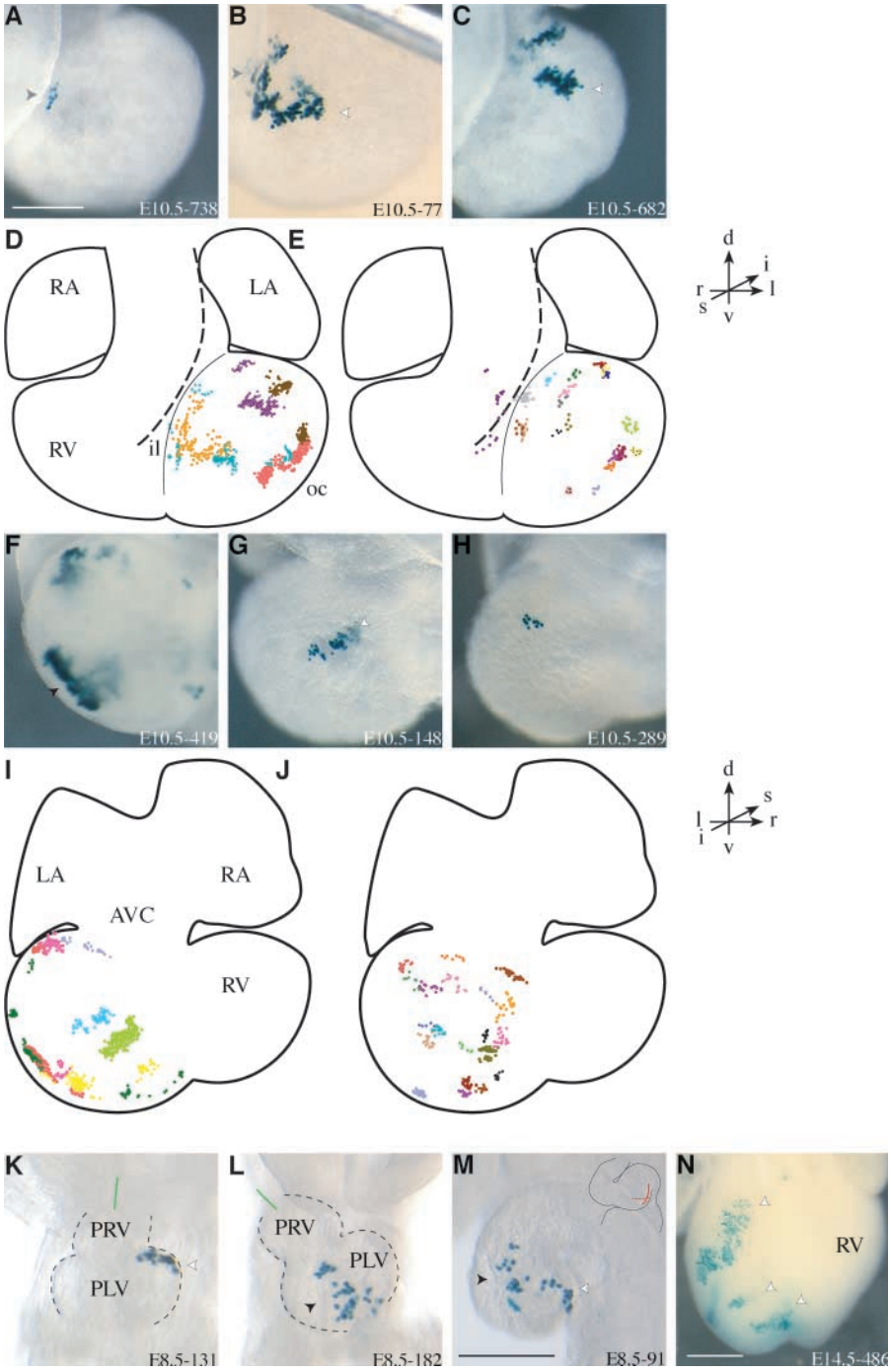

Figure 4. **Clusters of β-galactosidase–positive cells in the left ventricle.** Examples of staining in the embryonic left ventricle at E10.5 in a superior (A–C) and inferior (F–H) view, with a low (A and H) or high (B, C, F, and G) number of cells. Schematic representations of an E10.5 heart in the same views (D and E; I and J, respectively), showing the large (D and I) and small (E and J) clones that were observed in the embryonic left ventricle, each in a distinct color. The dotted contour in D and E represents the limit of the outflow tract; however, only β-galactosidase–positive cells, which are behind the outflow tract, are shown. The inner limit (il) of the left ventricular bulge is represented by a thin line. (K–M) Examples of large clones in the primitive left ventricle of the linear (K, superior view, stage 6 somites), early looped (L, superior view, stage 8 somites), and later looped (M, left lateral view, stage 12 somites) heart tube. The green lines in K and L point to the direction of the arterial–venous axis. The inset in M schematizes the orientation of the clusters (in red). (M) An example of a large clone in the left ventricle at E14.5 (inferior view). Arrowheads indicate a preferential orientation of β-galactosidase–positive cells at the inner limit of the bulging left ventricle (gray), along its outer curvature (black) and perpendicularly (white).

entations of clones are already found in this region at these early stages, shown in progressively more mature hearts as looping occurs (Fig. 4, K–M), both along the outer curvature of the arterial–venous axis (six cases; Fig. 4, L and M, black arrowheads) and perpendicular to it (nine cases; Fig. 4, K and M, white arrowheads). It is notable that these clones are already quite large. At E14.5, in the left ventricle, a similar orientation of larger clusters is observed (Fig. 4, compare N with G and I).

Therefore, the orientation of clonal growth in the left ventricle is discontinuous with neighboring regions; extension in perpendicular directions characterizes the bulging of this chamber from the cardiac tube. It is already initiated in the early linear cardiac tube and still reflects the expansion of the left ventricle after E10.5. As in the embryonic right ventricle, the orientation of clusters in small clones at E10.5 (Fig. 4, E and J), i.e., derived from a recent precursor, is less stereotyped and not always similar to that in large clones (Fig. 4, D and I), again suggesting that a secondary orientation event has begun before E10.5. Indeed, small clones may correspond to the secondary rows of cells found in larger clones (Fig. 4, compare H with G).

Atrioventricular canal and body of the atrium

Between the atrial appendages and the embryonic left ventricle at E10.5, there is a smooth-walled portion of the car-

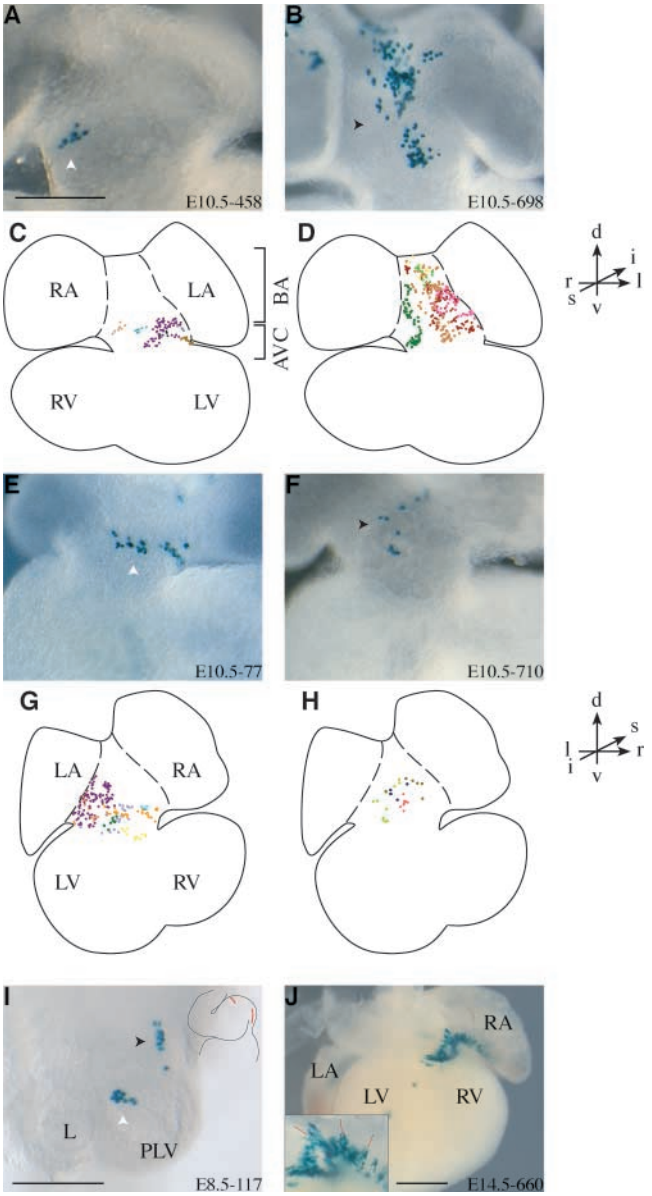

Figure 5. **Clusters of β-galactosidase–positive cells in the atrioventricular canal and the body of the atrium.** Examples of staining at E10.5 in superior (A and B) and inferior (E and F) views, with a low (A and F) or high (B and E) number of cells. Schematic representations of an E10.5 heart in the same views (C and D; G and H, respectively), showing the clones ($n \geq 5$ cells), each in a distinct color, that were observed in the atrioventricular canal (C and G) and in the body of the atrium (D and H). The potential limit between the atrioventricular canal (AVC) and the body of the atrium (BA) is indicated between C and D. Note the continuity of the clone represented in violet in the superior (C) and inferior (G) views. The dotted contour underlines the limit of the atrial appendages, which are not considered in this figure (see Fig. 6). In A–D, the outflow tract has been removed for direct visualization of labeled cells. (I) An example of a clone at the venous pole, between the primitive left ventricle and the sinus venosus, at E8.5 (rostral view). The orientation of clones (in red) is schematized in the inset. L, lumen of the arterial pole. (J) An example of a clone in the inlet of the right ventricle and in the smooth-walled lower rim of the atrium at E14.5 (inferior view). Inset in J shows a close-up of the clone, with the orientation of secondary rows of cells indicated by red bars. Arrowheads indicate a preferential orientation of β-galactosidase–positive cells, which is parallel (black) or perpendicular (white) to the arterial–venous axis.

diac tube. The atrioventricular canal contains cushion tissue and is adjacent to the embryonic left ventricle, whereas the body of the atrium, lying between the atrial appendages, contains the primary interatrial septum, which is only a primordium at this stage. Clonally related cells in the atrioventricular canal form lines with a circumferential orientation, both superiorly (Fig. 5, A and C) and inferiorly (Fig. 5, E and G). In contrast, in the body of the atrium, clusters of clonally related cells are elongated in a perpendicular orientation, i.e., along the arterial–venous axis (Fig. 5, B, D, F, and H).

At E8.5, the presumptive atrioventricular canal and the body of the atrium are in the portion of the cardiac tube between the primitive left ventricle and the sinus venosus (Moorman et al., 2003). In this area, clones have an axial orientation (14 cases; Fig. 4 I, black arrowhead). Occasionally, clusters are organized circumferentially near the primitive left ventricle, i.e., in the presumptive atrioventricular canal (three cases; Fig. 4 I, white arrowhead).

Between E10.5 and E14.5, the body of the atrium undergoes extensive remodeling during the process of septation of the atria, and is therefore difficult to distinguish at fetal stages. The atrioventricular canal is known to be integrated into the inlet of the ventricles (Moorman et al., 2003) as well as the smooth-walled lower rim of the atria (Franco et al., 2000). A clone was observed that colonized both these regions at E14.5 (Fig. 5 J) with a similar orientation to those seen at E10.5 in the atrioventricular canal (Fig. 5, compare J with E). However, this contains a higher number of cells, with extensive suborganization into secondary rows of cells (Fig. 5 J, inset).

These observations indicate that orientation of clonal growth in the presumptive atrioventricular canal and body of the atrium is detectable in the early cardiac tube, mainly reflecting the elongation of the cardiac tube. This persists in the body of the atrium at E10.5. In contrast, from E10.5 onwards, oriented clonal growth now clearly characterizes the enlargement of the atrioventricular canal and is accompanied later by the growth on a secondary axis, as seen already in the ventricles at E10.5.

Atrial appendages

The atrial appendages bulge out from the venous pole of the E10.5 heart. In the right atrial appendage, clonally related cells are organized into radiating lines (Fig. 6, A–E), converging toward an inferior dorsal pole (Fig. 6 A; Fig. 6 D, asterisk) or a ventral medial pole (Fig. 6 C; Fig. 6 E, asterisk). Small clusters follow the same orientation (Fig. 6, B, D, and E). In contrast, in the left atrial appendage, which is smaller than the right because bulging is delayed, fewer clones were obtained (Table I; Fig. 6, compare I and J with D and E). Clonally related cells appear more scattered, with an arched orientation, in relation to the atrium (Fig. 6, F–J).

At E8.5, the primitive atria are positioned where the venous pole of the cardiac tube bulges and bifurcates (Fig. 1 M), the left presumptive atrial region being smaller (Moorman et al., 2003). Distinct lines of a few clonally related cells are observed in the primitive right atrium (Fig. 6 K), whereas labeled cells extend along the circumference of the primitive left atrium (seven cases; Fig. 6 L).

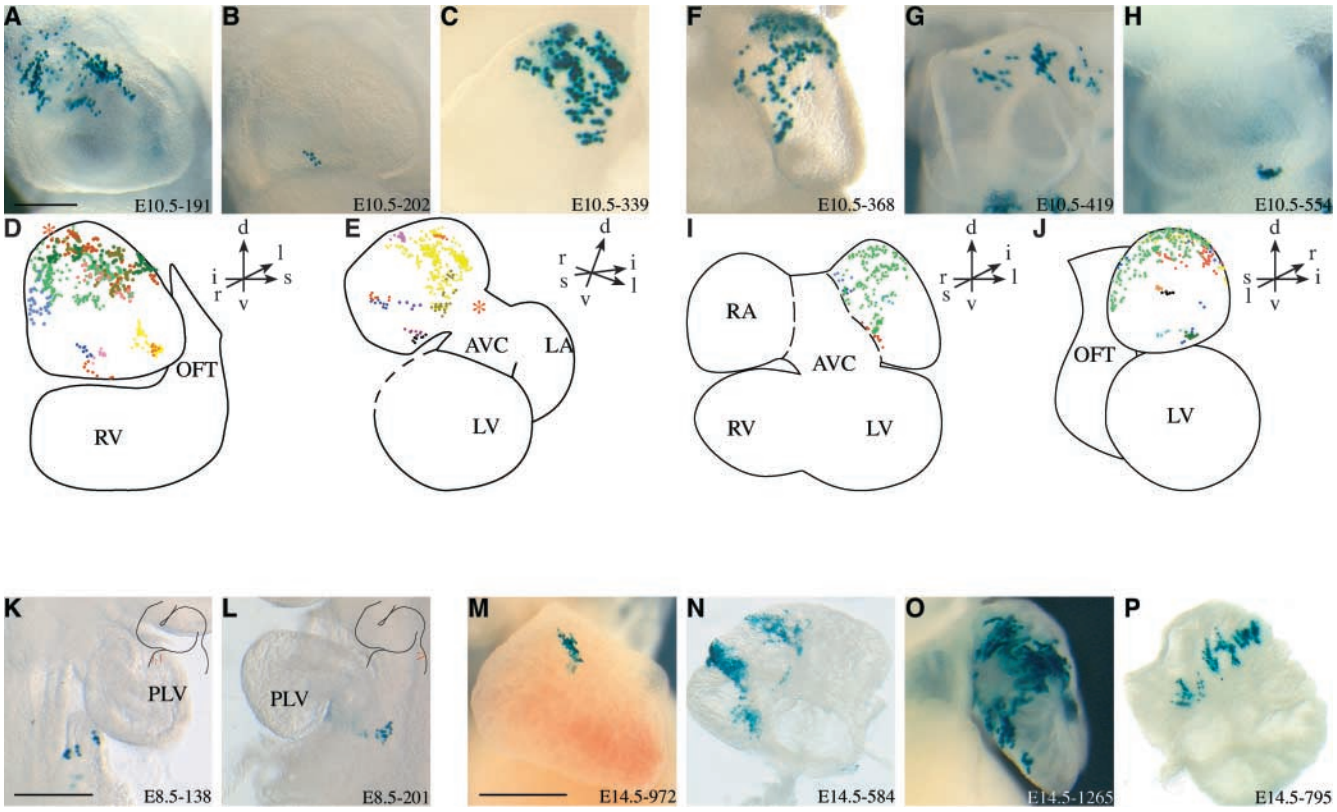

Figure 6. **Clusters of β-galactosidase–positive cells in the atrial appendages.** (A–E, K, M, and N) Right atrium. Examples of staining in the right atrial appendage at E10.5 in right lateral (A and B) and ventral (C) views, with a high (A and C) or low (B) number of cells. Schematic representations of an E10.5 heart in the same views (D and E, respectively), showing the clones ($n \geq 5$ cells) that were observed in the right atrial appendage, each in a distinct color. The dotted contour in E represents the site of section of the embryonic right ventricle and outflow tract that was removed to permit a direct visualization of the ventral side of the right atrial appendage. Asterisks indicate the point of convergence of the oriented clusters. (K) An example of a clone in the primitive right atrium at E8.5 (right lateral view). The orientation of the clone (in red) is schematized in the inset. (M and N) Examples of clones in the right atrium at E14.5 (M, lateral view; N, ventral view). (F–J, L, O, and P) Left atrium. Examples of staining in the left atrial appendage at E10.5 in superior (F) and left lateral (G and H) views, with a high (F and G) or low (H) number of cells. Schematic representations of an E10.5 heart in the same views (I and J, respectively), showing the clones that were observed in the left atrial appendage, each in a distinct color. The dotted contour in I indicates the limit of the atrial appendages. (L) An example of a clone in the primitive left atrium at E8.5 (left lateral view). The orientation of the clone (in red) is schematized in the inset. (O and P) Examples of clones in the left atrium at E14.5 (O, superior view; P, ventral view).

At E14.5, clusters of labeled cells are observed in the fetal right atrial appendage (Fig. 6, M and N) with a similar orientation to that at E10.5 (Fig. 6, compare M with B and N with C). Labeled cells in the fetal left atrial appendage (Fig. 6, O and P) also have a similar orientation to that at E10.5 (Fig. 6, compare O with F), and again they tend to be more dispersed than those in the right atrial appendage.

Therefore, orientation of clonal growth in the atrial appendages is discontinuous with the body of the atrium. Notably, different strategies of oriented cell growth characterize the bulging of the left and right atrial appendages, and this seems to already be the case at early stages. It is striking that the orientation of clones in the atrial appendages at E14.5 corresponds to the organization of trabeculations (pectinate muscles), which also differs between the two chambers (Sedmera et al., 2000). An additional orientation of clonal cell growth becomes obvious at E14.5, as shown by the presence of secondary rows of cells (Fig. 6 P).

Clone shapes can be generated by oriented mitosis

To investigate the mechanism by which clones acquire their shape, we have performed a computer simulation of myocardial cell growth. Cell division rate was taken as constant. The distance between cells was maintained by random rearrangement of their position after division. When the orientation of mitosis is random, i.e., when the position of daughter cells is random, clones display round shapes with no obvious orientation (Fig. 7 A). In contrast, when mitosis is oriented, i.e., when daughter cells tend to assume a particular orientation in relation to each other, either horizontally (Fig. 7 B) or radially (Fig. 7 C), clones adopt an elongated shape, similar to that observed in the left ventricle (Fig. 4 G) or left atrium (Fig. 6 A). Surprisingly, the main orientation of the clone is not strictly parallel to the orientation of mitosis, which is strictly horizontal in Fig. 7 B, indicating the role of post-mitotic cell rearrangement in modulating the orientation of clones. However, it is notable that clones conserve an orientation despite the subsequent random rearrangements of cells. Therefore, there is a clonal memory of the initial position of cells so that a constant constraint is not necessarily required to orient them. A low level of cell intercalation is also observed between labeled and unlabeled cells (Fig. 7 B) or between clones (Fig. 7 C), which reflects the biological observations. This modeling shows that orientation

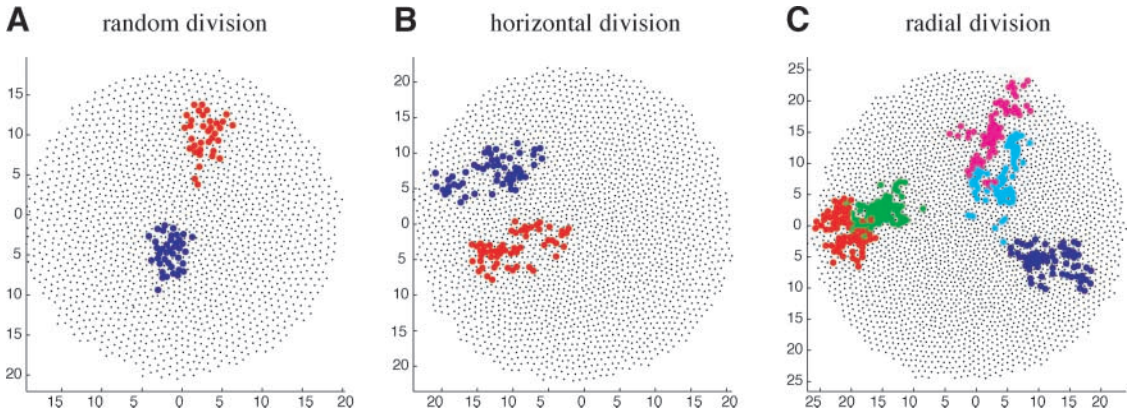

Figure 7. **Modeling of myocardial cell growth.** Cells are represented by dots and are assumed to divide with a constant rate. They undergo random rearrangements after division to maintain their relative distance constant. The position of daughter cells is oriented either randomly (A), horizontally (B), or radially (C). Clones of cells are labeled, each in a distinct color.

of mitosis would be sufficient to account for the generation of clone shapes. This is in agreement with observations in the zebrafish (Kimmel et al., 1994), and suggests that complex signaling mechanisms mediating oriented growth, such as chemioattraction between cells, are not necessarily required.

Discussion

The approach we have adopted to clonal analysis of the mouse myocardium is based on the spontaneous activation of a reporter gene, and has therefore permitted us to generate a random sampling of clones. This is the first time that long-term clonal growth of myocardial cells has been monitored. Compilation of data on the shape of clones demonstrates that clonal growth is dynamic and oriented in relation to heart morphology. This analysis was mainly based on observations at E10.5, when the mouse heart is still a tube, but the future compartments are clearly distinguishable. We show that the orientation of clones is a specific characteristic of each cardiac region, as summarized in Fig. 8 (A and B). Furthermore, this appears to be maintained in the different compartments of the fetal heart at E14.5 and to be already detectable in the early embryonic heart tube. These observations suggest that oriented clonal cell growth is a key component in heart morphogenesis, and underlies the development of the cardiac chambers.

Cellular basis of heart tube regionalization

The differences in clonal orientation indicate that cardiac regions can be distinguished on the basis of cell behavior. Previously, distinction of future cardiac compartments along the arterial–venous axis of the cardiac tube was based on their morphology and histology (Moorman et al., 2003), their electrophysiological properties (Moorman et al., 1998), and their expression of endogenous genes (Franco et al., 1998) and transgenes (Kelly et al., 1999). The regions identified here are similar, but reveal further subtleties. The pattern of clonal cell growth clearly demarcates the body of the atrium from the atrial appendages and from the region of the future atrioventricular canal, for example. It also shows that the proximal outflow tract has similar growth properties to the embryonic right ventricle, prefiguring its incorporation into this chamber as the heart matures (Franco et al., 1997). The interventricular region has specific characteristics of oriented cell growth, and thus constitutes a distinct region of the myocardium.

If one considers the common origins of certain regions of the heart predicted from models for cardiac morphogenesis, the orientation of clonal growth does not reflect this parameter. Thus, clones in the outflow tract and right ventricle, potentially both derived from the anterior heart field (Kelly et al., 2001; unpublished data), have distinct growth characteristics, as do clones in the working myocardium of the different cardiac chambers or in different parts of the primary myocardium of the cardiac tube (Christoffels et al., 2000a). Oriented cell growth is directly correlated with the way in which each part of the cardiac tube expands to form the four-chambered heart.

Within a region, clonal growth appears to be modulated differently in opposing parts of the tube (Fig. 8 C); cell density of clones is higher superiorly than inferiorly in the outflow tract, and clusters in the right ventricle are discontinuous dorsally and ventrally, radiating out from different points, whereas in the left ventricle, clusters situated superiorly and inferiorly are discontinuous. Discontinuity is obvious in the largest clones, derived from earlier precursors, as illustrated by clone E10.5–554 (Fig. 2, D and I; green), which is probably derived from a precursor labeled at the time of gastrulation. Still, this contribution to the right ventricle is discontinuous in the ventral and dorsal aspects. Such regionalization, summarized in Fig. 8 C, is also reflected by the expression of the homeobox transcription factor Pitx2c (Campione et al., 2001), which is detected in the superior region of the outflow tract, the ventral right ventricle, the inner curvature of the left ventricle, and the left atrium. It has been proposed in the chick that this regionalization dates back to the primitive bilateral separation of cardiac precursor cells, when they are displaced from the primitive streak to the anterior of the embryo. The right and left regions of the splanchnic mesoderm give rise to the right and left components of the cardiac crescent, and then of the linear cardiac tube (Stalsberg, 1969). The right/left distinction becomes transposed as the tube loops to inferior/superior for the out-

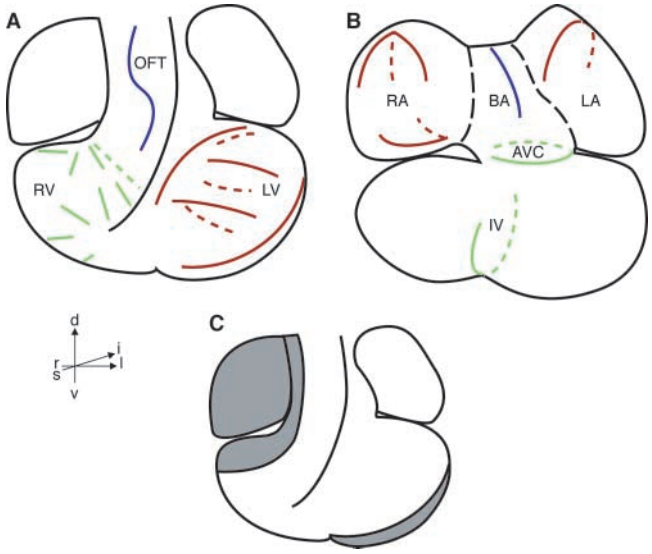

Figure 8. **Orientation of clonal growth in the embryonic heart.** (A and B) Schematic representations of an E10.5 heart in a superior view, showing a summary of the orientation of clonal growth presented in this paper. Continuous lines indicate clonal growth in a superior view and dotted lines in an inferior view. For clarity, data in the outflow tract (OFT), embryonic right ventricle (RV), and embryonic left ventricle (LV) are illustrated in A, and in the interventricular region (IV), atrioventricular canal (AVC), body of the atrium (BA), and atrial appendages (LA, RA) in B, in which the outflow tract has been removed. Based on the shape of clusters of clonally related cells, three patterns of clonal growth are distinguished. Clonal growth has a preferential axial orientation along the arterial–venous axis (in blue) in the distal outflow tract and in the body of the atrium. Note the spiralling of the axis in the outflow tract. Clonal growth is circumferential (in green) in the proximal outflow tract, embryonic right ventricle, interventricular region, and atrioventricular canal. In the embryonic left ventricle and the atrial appendages, which bud out from the tube, clonal growth has a more complex orientation (in red): arching over the circumference in the left atrium, lying radially in the right atrium, and showing two perpendicular orientations in the left ventricle. d, dorsal; i, inferior; l, left; r, right; s, superior; v, ventral. (C) Regionalization of the orientation of clonal growth within a given region. Cell behavior is distinct (gray/white) between the inferior/superior aspects of the outflow tract and between the dorsal/ventral right ventricle as well as between the right and left atria, and it is discontinuous in the inferior/superior parts of the left ventricle.

flow tract and left ventricle and to ventral/dorsal for the right ventricle. Thus, the regionalization of cell behavior within cardiac regions, as well as the distinct cell behavior between left and right atrial appendages that we report here, may reflect at least in part the early left/right separation of myocardial cell precursors.

Dynamics of oriented clonal cell growth

Oriented clonal cell growth is already observable in the early cardiac tube, suggesting that a proportion of cells have a regional identity at this stage. Left ventricular characteristics are clearly detectable at the linear heart tube stage, whereas in contrast, the orientation of clonal growth in the future atrial appendages is only beginning to emerge in the looped cardiac tube. This is consistent with the idea that in the mouse, unlike the chick (de la Cruz et al., 1989), the presumptive left ventricle is the major region present in the early cardiac tube (unpublished data), whereas the presumptive atrial region is added later (Viragh and Challice, 1973). We have observed shifts in the orientation of clonal cell growth from axial to circumferential in the atrioventricular canal, the proximal outflow tract, or the dorsal right ventricle, reflecting the change from a global elongation of the cardiac tube to more specific modes of regional expansion. Orientation of clonal cell growth is still observable in the E14.5 heart. However, modulations appear, such as secondary orientations within large clones, or novel orientations of small clones. These are perceptible as early as E10.5 in the ventricles, but only later in the atrioventricular canal and atrial appendages. Previously, we have shown that the secondary orientation of clonal cell growth prefigures myofiber architecture of the heart (Meilhac et al., 2003).

The interventricular region: a boundary?

Analysis of oriented clonal cell growth reveals the existence of a new, interventricular region with specific characteristics. This region has not been considered previously as a distinct segment in the prospective fate maps (Stalsberg and De Haan, 1969; de la Cruz et al., 1989), nor as a distinct transcriptional domain (Franco et al., 1998; Kelly et al., 1999). However, molecular markers can be found that are positive in this region and negative in the ventricles, such as *Irx1-2* (Christoffels et al., 2000b), or inversely negative in this region, and positive in the ventricles, such as *ANF* and *eHAND* (Christoffels et al., 2000a).

The interventricular region is situated at the boundary between the right and left ventricles. Our results indicate that a circumferential orientation of clonal cell growth, in relation to the cardiac tube, appears in this region at ~E8.5, before the formation of the interventricular septum. This is reminiscent of clonal cell growth at the vein margin in the *Drosophila* wing (Resino et al., 2002), which parallels this compartment boundary. Therefore, orientation of clonal growth in the interventricular region may well act as a barrier to cell mixing between the two ventricles, and may explain why left ventricular clusters do not expand into the right ventricle, and vice versa (unpublished data).

We show that clones in the interventricular region participate in the formation of the interventricular septum, as early as this is detectable morphologically (E10.5). Clones in this region extend into either left or right ventricular myocardium, suggesting that the interventricular septum has a dual origin rather than arising from left ventricular myocardium alone. The regionalized expression of certain transgenes (Franco, D., and R. Kelly, personal communication) and genes such as *Tbx5* (Bruneau et al., 1999) is consistent with both a left and right ventricular contribution. In contrast to the atrioventricular region, in which clonal growth has a circumferential orientation and underlies the enlargement of this portion of the cardiac tube, the interventricular region, in which clonal growth shows a similar orientation, forms a groove (i.e., its expansion is reduced compared with that of the adjacent ventricles). The invagination of cells into the septum may explain why the circumferential orientation

does not lead to an important enlargement of the interventricular region. Such an invagination process is consistent with the finding that quiescent cells are concentrated in the chick interventricular septum (Thompson et al., 1990).

Oriented clonal cell growth shapes the heart

Our work demonstrates that the myocardium is not only a regionalized tissue, but is also a polarized tissue. The orientation of clonal cell growth, which we identify here at E10.5 (Fig. 8, A and B), throws new light on how the cardiac tube expands during morphogenesis of the heart. Axial orientation, underlying the elongation of the cardiac tube, is observed at both poles (i.e., in the outflow tract and the body of the atrium). This is consistent with the well-established idea that elongation of the cardiac tube proceeds by addition of myocardial cells at both ends in chick (de la Cruz et al., 1989) and mouse (Viragh and Challice, 1973; Kelly et al., 2001). Previously, we have shown that the elongation of the rest of the cardiac tube is also characterized by an underlying axial orientation during the earlier phase of dispersive growth of myocardial precursor cells (Meilhac et al., 2003). In contrast, circumferential orientation of clonal growth reflects the enlargement of the cardiac tube at other sites, notably in the atrioventricular canal. Such growth provides a cellular basis for the expansion of the right atrioventricular junction, when the atrioventricular canal, initially continuous with the embryonic left ventricle, develops its connection with the right ventricle (Webb et al., 1998). Our results are consistent with the view that the cardiac chambers form by bulging from the outer curvature of the heart tube, proposed on the basis of morphology and gene expression patterns (Christoffels et al., 2000a). However, cardiac chambers adopt different growth strategies to achieve their final form (Fig. 8, A and B; red). Surprisingly, the right ventricle, unlike the other cardiac chambers, does not appear as a bulging structure. On the contrary, the orientation of clonal growth is circumferential, emanating from the inner curvature of the tube, which is bent back on itself as a result of cardiac looping. This indicates that the right ventricle forms by enlargement of the tube. Other aspects of heart morphogenesis, such as cardiac looping, are not reflected by the orientation of clonal growth; another facet of cell behavior, the change of their shape, may well intervene here (Manasek et al., 1972).

Differential rates of proliferation have been reported during development of the avian cardiac tube based on BrdU incorporation (Thompson et al., 1990), which was lower in the inner curvature, the outflow tract, and the atrioventricular canal compared with the rest of the cardiac tube, and lower in the left compared with the right atrium. This would explain why cardiac chambers bulge out from the outer curvature and why certain regions are smaller. However, differences in overall growth rates are not sufficient to explain why regions adopt a particular structure. In our work, small and large clones appear ubiquitously distributed within a region, which argues against the existence of proliferative centers governing chamber expansion (Rychter et al., 1979). The results presented here point to the orientation of clonal cell growth as a key parameter of cardiac chamber expansion. Analyses in other systems, like the *Drosophila* wing (Resino et al., 2002) or the *Antirrhinum* petal (Rolland-Lagan et al., 2003), support the hypothesis because their morphogenesis largely depends on oriented cell growth rather than on regional differences in growth rates. We now provide the first example of this phenomenon during mammalian morphogenesis.

Materials and methods

Production of clones

Random clones of myocardial cells, positive for β-galactosidase, were generated spontaneously using the mouse line α_c-actin$^{+/nlaacZ1.1}$ as described previously (Meilhac et al., 2003). To increase the number of transgenic embryos per litter, we have rescued the lethality of homozygous α_c-actin$^{nlaacZ1.1\Delta Neo/nlaacZ1.1\Delta Neo}$ mice, in which the *neomycin* (*Neo*) selection gene has been removed from the targeted allele, by crossing with an *αMHC-α$_c$-actin* transgenic line that expresses a cDNA for rat *α$_c$-actin* under the control of the *αMHC* promoter (a gift from A. Kumar and J. Lessard, Children's Hospital Medical Center, Cincinnati, OH). Rescued [α_c-actin$^{nlaacZ1.1\Delta Neo/nlaacZ1.1\Delta Neo}$ × *αMHC-α$_c$*-actin] males, which are viable and fertile, were crossed with superovulated wild-type females ([C57BL/6JxSJL]F1), as described previously (Meilhac et al., 2003), to generate additional α_c-actin$^{+/nlaacZ1.1\Delta Neo}$ embryos with clones of myocardial cells positive for β-galactosidase. The analysis here is based on 233 and 734 embryos and 595 fetuses dissected respectively at E8.5, E10.5, and E14.5, and which have β-galactosidase–positive myocardial cells. At E8.5, most embryos were at the looped heart tube stage (9–17 somites); however, due to asynchronous development, a few earlier embryos were also recovered at the linear heart tube stage (6–7 somites) and at intermediate stages of cardiac looping.

Clonal relationship between β-galactosidase–positive cells at E10.5 and the age of clones

At E8.5, due to the low frequency of positive hearts (7.5%; Meilhac et al., 2003), the double-recombination events that may occur in a single heart are negligible. Therefore, β-galactosidase–positive cells in an E8.5 heart are clonally related. Later, the frequency of positive hearts is higher (67% at E10.5), and double-recombination events may occur in a single heart. However, such contamination can be monitored because it corresponds to a recombination event in a late precursor after E8.5. Indeed, 90% of the E10.5 hearts contain <17 labeled cells. Because recombination is random, there is a low probability that a second recombination event occurs in the same location as the first; therefore, a cluster very probably contains clonally related cells. As an example in the outflow tract at E10.5 (Table I), the observed frequency of large clusters (0.6%), is significantly different ($\chi^2 = 143$, 1 degree of freedom, $P < 0.01$) from the expected frequency of two independent small clusters (0.03%).

Because myocardial cells and their precursors follow a proliferative growth mode and only a small proportion of these cells are apoptotic or quiescent at embryonic stages (Meilhac et al., 2003), the overall number of β-galactosidase–positive cells was taken as an indication of when recombination of *nlaacZ* to *nlacZ* had taken place during embryogenesis. Two categories of hearts were considered at E10.5; those with more than or those with less than 40 labeled cells in a given cardiac region (Table I). In the first category, labeled cells are either present in a single large cluster or in multiple clusters that are close to one another and/or aligned along the arterial–venous axis of the cardiac tube. This indicates that they derive from a common precursor, which was labeled early during the dispersive phase when multiple clusters are observed (Meilhac et al., 2003). Such clusters are referred to as "large clones." In the second category, which is much more frequent, labeled cells are distributed into small clusters that cannot be clonally related because of the high frequency of observation of small clusters. This suggests that each cluster derives from a recent precursor that was labeled during the coherent phase, and these are referred to as "small clones."

Schematic representation of cluster shapes at E10.5

Photographs of myocardial clones were superimposed on a schematic representation of the heart using Adobe Illustrator® software, with each β-galactosidase–positive nucleus in a heart represented as a colored dot. For clarity, individual regions are documented; any given positive heart may have more than one positive region. For better three-dimensional visualization, two different views of each cardiac region are shown. These were

chosen to best represent the large clones, which cover a larger area. The anatomical axes (dorsal/ventral, superior/inferior, left/right) used for the description are defined in Fig. 1. We use the nomenclature superior/inferior to show the correspondence between the cardiac structures at different stages. Due to looping of the heart, the orientation of the superior/inferior axis varies in relation to the body. It corresponds to the left/right axis of the body at the linear heart tube stage, to the ventral/dorsal axis at the looped stage, and then approaches the rostral/caudal axis in the late embryonic heart. Labeled clusters containing <5 cells had no obvious orientation and were discarded in this analysis. The orientation of clonal growth at E10.5 and E14.5 was documented to describe the surface expansion; therefore, the orientation of deeper clusters in the ventricles was not taken into account in this analysis.

Virtual transverse sectioning by optical projection tomography

Hearts with β-galactosidase–positive cells from E10.5 embryos were prepared, autofluorescence was scanned using a GFP1 filter (Leica), and images were reconstructed as described previously (Sharpe et al., 2002).

Modeling of clone shapes

The simulations were performed using the MATLAB® 6.5 technical computing system (The MathWorks, Inc.). Cells are modeled as "nodes" connected to six nearest neighbors by nonlinear springs with fixed resting length $L = 1$. The cells initially form a regular array (or "lattice") in a plane. They are subjected to (weak) random forces, and may rearrange extensively. In simulated mitosis, (1) a mitotic axis is first defined, which is either taken as horizontal, oriented at random, or pointing toward the center of the array (0,0); (2) the mother cell disappears and is split into two daughters, each located at a distance L/2 from the mother. The daughters' 6 + 6 nearest neighbors are determined and new springs are introduced; and (3) the network of springs is allowed to relax (i.e., the daughters "make space" for themselves) before another cell undergoes mitosis. The relaxation process slows down with the increasing number (n) of cells, and for this reason the network may become irregular; most notably, density increases near the center. This would only disappear after a prohibitively long computation time. For computational convenience, the initial array comprises $n = 49$ cells on a square lattice with x and y coordinates spanning the range -3 to $+3$; however, all traces of the square lattice quickly disappear as dividing cells spontaneously arrange themselves in an approximate, imperfect honeycomb (hexagonal) pattern. After all the original cells have undergone their first mitosis, some cells are marked in color, and their descendents all inherit that color, thus forming a visible clone. All the simulations shown were stopped when the cells numbered about $n = 3{,}000$ (i.e., after ~5–6 rounds of mitosis). Each simulation took ~90 min on a workstation (Ultra60; Sun Microsystems, Inc.).

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