Biphasic Development of the Mammalian Ventricular Conduction System

Lucile Miquerol, Natividad Moreno-Rascon, Sabrina Beyer, Laurent Dupays, Sigolène M. Meilhoc, Margaret E. Buckingham, Diego Franco, Robert G. Kelly

Rationale: The ventricular conduction system controls the propagation of electric activity through the heart to coordinate cardiac contraction. This system is composed of specialized cardiomyocytes organized in defined structures including central components and a peripheral Purkinje fiber network. How the mammalian ventricular conduction system is established during development remains controversial.

Objective: To define the lineage relationship between cells of the adult ventricular conduction system and surrounding working myocytes.

Methods and Results: A retrospective clonal analysis using the α-cardiac actin^{nlaacZ+} mouse line was carried out in three week old hearts. Clusters of clonally related myocytes were screened for conductive cells using connexin40-driven enhanced green fluorescent protein expression. Two classes of clusters containing conductive cells were obtained. Mixed clusters, composed of conductive and working myocytes, reveal that both cell types develop from common progenitor cells, whereas smaller unmixed clusters, composed exclusively of conductive cells, show that proliferation continues after lineage restriction to the conduction system lineage. Differences in the working component of mixed clusters between the right and left ventricles reveal distinct progenitor cell histories in these cardiac compartments. These results are supported by genetic fate mapping using Cre recombinase revealing progressive restriction of connexin40-positive myocytes to a conductive fate.

Conclusions: A biphasic mode of development, lineage restriction followed by limited outgrowth, underlies establishment of the mammalian ventricular conduction system. (Circ Res. 2010;107:153-161.)

Key Words: conduction system • cell lineage • clonal analysis • nlaacZ • Purkinje fibers

The cardiac conduction system controls the generation and propagation of electric activity through the heart to coordinate cardiac contraction. Atrial contraction is initiated by the sinoatrial node or pacemaker. After a delay mediated by the atrioventricular node (AVN), the ventricular conduction system (VCS) ensures rapid propagation of electric activity to the ventricular apex. The VCS is comprised of a central component, the atrioventricular (AV or His) bundle and right and left bundle branches, and a peripheral component composed of a dense ellipsoid network of Purkinje fibers. These structures have been well characterized in adult mouse and human hearts by their specific histological and electrophysiological properties. However, despite the clinical importance of the VCS in regulating cardiac rhythm, important questions remain as to the origin and the mode of development of the mammalian VCS.

Existing views of VCS development are largely based on data obtained in avian embryos, despite major anatomic differences within the VCS between birds and mammals. In the avian system, lineage analysis using replication defective retroviral labeling has demonstrated that conductive cells share common progenitors with working cardiomyocytes and that the VCS develops by a process of induction and recruitment of myocytes through endothelial derived signals. According to this model, conductive myocytes are nonproliferative and subsequent growth of the conduction system occurs by accretional recruitment of new myocytes (or ingrowth). However, in the chick, a perivascular network of Purkinje fibers is localized deep in the myocardium in proximity to coronary vessels, whereas in the mouse, as in humans, Purkinje fibers are present only at the subendocardial ventricular surface and it is unclear whether the chick model is applicable to the mammalian VCS. In mammals an outgrowth model has been proposed for development of the central VCS, by which conductive cells develop independently of the working myocardium from a pool of conductive...
progenitors at the crest of the interventricular septum (IVS) in the early embryonic heart.9–11 Tbx3, encoding a T-box containing transcription factor, has been identified as an early marker of the central VCS and is required for the development of the AVN and AV bundle in the mouse.12 Outgrowth of the murine central VCS is supported by the observation that bundle branch myocytes appear to be derived in part from a Mesp1-negative cell population.13 However, no lineage data are available concerning development of the mammalian Purkinje fiber network and the extent to which ingrowth versus outgrowth models contribute to establishment of different components of the mammalian VCS remains highly controversial.

Here, we use retrospective clonal analysis of the murine VCS to address these questions. Retrospective clonal analysis consists of studying the properties of clonally related cells such as clone size, cell identity and cell dispersion to provide information on the events that take place during development of a particular structure. In mammals, retrospective clonal analysis using the nlacZ reporter gene has been demonstrated to be a powerful technique for analyzing cell behavior during organogenesis.14,15 This approach is based on a defective reporter gene carrying a duplication with a stop codon in the β-galactosidase coding sequence. Low frequency spontaneous intragenic recombination leads to loss of the duplication and restores the nlacZ open reading frame. Cardiac specific expression of the nlacZ gene under transcriptional control of the α-cardiac actin locus allows retrospective clonal analysis of myocardial cells in the mouse heart.16 Extensive studies of collections of mosaic nlacZ/lnlacZ embryonic hearts have made significant contributions to the understanding of cardiac morphogenesis.17–20

Here, we have used the nlacZ system coupled with a reporter transgene to study the clonal relationship between conductive and working cardiomyocytes in the mouse. α-cardiac actinnlacZ/lnlacZ mice have been crossed with connexin (Cx)40GFP/ mice in which the entire ventricular conduction system can be visualized through activity of an eGFP reporter gene.21 Our results provide new insights into the origin and establishment of the VCS and reveal that outgrowth follows specification during development of the mammalian Purkinje fiber network. This conclusion is supported by genetic fate mapping using a novel inducible Cre allele at the Cx40 (Gja5) locus. Induction of Cre activity at different developmental time points confirms the biphasic nature of VCS development and suggests that lineage restriction between trabecular cardiomyocytes and the Purkinje fiber network is largely complete by embryonic day (E)16.5.

Methods

Animal care was in accordance with national and institutional guidelines. Double transgenic knock-in α-cardiac actinnlacZ/lnlacZ::Cx40eGFP mice were crossed with CD1 females to generate nlacZ+/−/Cx40eGFP mice for the production of β-gal clones. The α-cardiac actinnlacZ−/− and Cx40eGFP+ mouse lines used in this study were previously reported.16,21 We have generated a Cx40-CreERT2 mouse line for lineage tracing experiments (Beyer et al in preparation). A Cre-ERT2-IRESmRed reporter cassette12,23 has been inserted at the Cx40 locus by homologous recombination using standard techniques. 4OH-Tamoxifen (H7904, Sigma) was injected intraperitoneally into pregnant females carrying the Cx40-CreERT2 cassette and the R26R reporter gene24 at different time points.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Analysis of nlacZ Clusters in the Ventricular Conduction System

Optical projection tomography was used to visualize the VCS in three week old Cx40GFP/+ hearts (Figure 1 and Online Movie 1). The VCS is fully differentiated at this stage and can be divided into central and peripheral components. The central VCS comprises the AV (His) bundle localized at the crest of the IVS, the left bundle branch (LBB) in the upper half of the left part of the septum and the right bundle branch (RBB) descending along the right septal surface. The peripheral VCS comprises left ventricular Purkinje fibers (LPF) forming a complex network covering the subendocardial surface of the left ventricle and right ventricular Purkinje fibers (RPFs) forming a small network on the right side of the septum connected to a more extensive network on the subendocardial surface of the right ventricular free wall. Calculation of the relative volume of GFP-positive cells at this stage reveals that the VCS represents 1.2% of ventricular myocardium.

We subsequently analyzed the clonal relationship between GFP-positive cells of the VCS and surrounding GFP negative myocytes. Our study is restricted to the VCS as Cx40 is not expressed in the sinoatrial node. Specifically, we investigated whether clonally related cardiomyocytes in three week old α-cardiac actinnlacZ−/−::Cx40eGFP hearts were restricted to the VCS or labeled both the VCS and adjacent working myocytes. α-cardiac actinnlacZ−/−::Cx40eGFP/ mice were generated by breeding α-cardiac actinnlacZ−/−::Cx40eGFP/ males with nontransgenic females. 3 week old hearts were dissected to analyze the distribution of β-galactosidase–positive cells in the conduction system. 100% of hearts contained β-galactosidase–positive cells, indicating that several independent recombination events may have occurred in
the majority of α-cardiac actin<sub>α-actin</sub> hearts by three weeks, resulting in multiple β-galactosidase–positive clusters in the same heart. We restricted our analysis to individual clusters, defined as compact groups of β-galactosidase–positive nuclei. The number of visible clusters varied and on average 10 clusters were observed per heart. Approximately 9000 β-galactosidase–positive clusters were obtained from 894 three week old hearts, of which 525 were preselected as being potentially in the VCS based on location at the subendocardial surface of the ventricles, overlapping with or close to GFP-positive cells (Figure 2). Of these 525 clusters, 82 have been definitively shown to contain GFP-positive cells after sectioning and immunostaining with an anti-GFP antibody (Table 1). Thus less than 1% of visible clusters (82/9000) are located in the VCS, consistent with the small proportion of conductive relative to working ventricular myocytes. 90% (74/82) of these clusters are found in separate hearts and only 4 hearts were scored with two clusters in the VCS. To assess whether two VCS clusters found in the same heart result from independent recombination events, we have compared the observed distribution of the number of clusters in the VCS per heart with the expected distribution of independent recombination events per heart, based on the Luria and Delbrück fluctuation test (Table 2). A frequency of 4 of 82 is consistent with the occurrence of two independent recombination events resulting in VCS labeling in the same heart.

Table 1. No. and Properties of Clusters Analyzed

<table>
<thead>
<tr>
<th>Type of clusters</th>
<th>Total no. of hearts</th>
<th>IVS†</th>
<th>RV‡</th>
<th>LV§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of hearts with X-gal–positive clusters</td>
<td>894</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. of conductive clusters&lt;sup&gt;+&lt;/sup&gt; (percentage of conductive clusters&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>82 (0.95%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of mixed clusters</td>
<td>33</td>
<td>9</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>No. of unmixed conductive clusters</td>
<td>49</td>
<td>12</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>No. of subendocardial unmixed working clusters&lt;sup&gt;+&lt;/sup&gt; (sectioned)</td>
<td>443 (144)</td>
<td>164</td>
<td>162</td>
<td>199</td>
</tr>
</tbody>
</table>

<sup>†</sup>Conductive clusters in the IVS are in the central VCS (His, RBB, LBB). <sup>‡</sup>Conductive clusters in the right ventricle are in RPFs. <sup>§</sup>Conductive clusters in the left ventricle are in LPFs.

**Figure 1.** Three-dimensional reconstruction of the ventricular conduction system by optical projection tomography. A, Ventral view of a 3D volume rendering of anti-GFP whole-mount immunostaining of a 3-week-old Cx40<sub>α-GFP</sub> mouse heart. B, Three-dimensional view of the VCS inside the left ventricle (LV). **Red arrow** indicates His bundle; **white arrows**, LBB; **red arrowheads**, LPF. C, Three-dimensional view of the right side of the IVS (RS). **Red arrow** indicates His bundle; **red arrowhead**, RBB; **asterisk**, RPF. D, Three-dimensional view of the RPF network (**asterisk**) on the right ventricular free wall (RV).

**Figure 2.** Visualization of double positive β-gal/ GFP clusters in different units of the ventricular conduction system. A through E, Superposition of fluorescent and bright field whole-mount images from double-positive β-gal/GFP clusters. A, Atrioventricular bundle (His). B and C, RBB and LBB. D and E, RPF and LPF. **Arrowheads** indicate GFP expression in endothelial cells of coronary vessels.
Table 2. Frequency of Hearts With N Recombination Events in the VCS

<table>
<thead>
<tr>
<th>N</th>
<th>No. of Hearts With N Recombination Events in the VCS, Expected*</th>
<th>No. of Hearts With N Clusters in the VCS, Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>816</td>
<td>816</td>
</tr>
<tr>
<td>1</td>
<td>74.49</td>
<td>74</td>
</tr>
<tr>
<td>2</td>
<td>6.80</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>0.62</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0.005</td>
<td>0</td>
</tr>
</tbody>
</table>

*From the no. of hearts without clusters in the VCS (816), the no. of hearts presenting N recombination events in the VCS (from 1 to 4) was calculated using the fluctuation test of Luria and Delbrück.

(Table 2). This analysis strongly suggests that each β-galactosidase–positive cluster containing GFP-positive cells results from an independent recombination event and that cells within these clusters can be considered to be clonally related, consistent with the coherent growth of myocardial cells.16 18 Additional hearts, containing multiple large β-galactosidase–positive clusters of several thousand cells, are likely to result from early recombination events and were excluded from the present analysis.

Two Classes of Clusters: Mixed and Unmixed

The 82 conductive clusters containing β-galactosidase and GFP-positive cells were divided into two classes, mixed and unmixed, depending on their cellular composition. Mixed clusters contain both GFP-positive and GFP-negative cells (Figure 3A, 3A′, and 3A″). The existence of both conductive and working myocytes within single β-galactosidase–positive clusters shows that the labeling event occurred in a precursor cell before lineage restriction to conductive or working myocardium. Such cells, of which only a fraction of descendants give rise to the VCS, correspond to the ancestral cells of the VCS. In contrast, unmixed conductive clusters contain only GFP-positive cells (Figure 3B, 3B′, and 3B″) and result from clonal growth of a cell already restricted to a conductive fate, corresponding to a founder cell of the VCS or one of its descendants. In addition to these 82 clusters, 443 unmixed GFP-negative clusters were observed in proximity to the subendocardial ventricular surface (Figure 3C, 3C′, and 3C″), resulting from the clonal growth of working cardiomyocytes.

The number of clusters observed as a function of cluster size is presented in Figure 4 for each class of cluster. Unmixed conductive clusters are small and contained a maximum of 30 cells; approximately 80% of such clusters were composed of less than 10 cells (Figure 4A). Analysis of cluster frequency as a function of size can provide insights into growth modes: under conditions of proliferative growth, the number of cells doubles at each division and the frequency of clones thus decreases exponentially with increasing clone size.15 We observed that this is the case for unmixed conductive clusters consistent with a proliferative mode of conductive cell outgrowth (coefficient of regression $R^2=0.91$). Unmixed working clusters, representative of the working myocardium, also follow a proliferative growth mode (Figure 4B, $R^2=0.96$). The limited size (mean cluster size, 6 cells) of unmixed conductive clusters suggests that the proliferative growth of conductive cells is restricted to 4 to 5 cell divisions. In contrast, proliferative growth is more extensive in working myocytes and the mean cluster size of unmixed working clusters is 24 cells.

Figure 3. Identification of 2 classes of clusters: mixed and unmixed. A, Mixed conductive clusters: β-gal–positive cells are distributed in the conductive (GFP+) and working myocardium (GFP−). B, Unmixed conductive clusters: all β-gal+ cells are GFP+ and restricted to the conduction system. C, Unmixed working clusters: β-gal+ cells are localized in the working myocardium (GFP−). A through C, Whole-mount images showing β-gal and GFP expression. A′ through C′, Immunohistochemistry analyses with an anti-GFP antibody on sections of corresponding clusters in A through C enable identification of the different classes of clusters. A′ through C′, Higher magnification of sections in A′ through C′. Scale bars: 100 μm (A′ through C′); 25 μm (A′ through C′).
Mixed clusters containing GFP-positive and GFP-negative cells arise in the ancestral cells of the VCS, which give rise to both conductive and working myocytes. In contrast to unmixed clusters, mixed clusters contained at least 5 cells and the majority (65%) more than 30 cells (Figure 4C). The size of β-galactosidase–positive clusters depends on the number of cell divisions that have occurred since the intragenic recombination event. Mixed clusters, which tend to be large, have undergone more cell divisions and are thus likely to be born earlier in development than unmixed conductive clusters. The frequency of mixed clusters is approximately equivalent over a range of cluster sizes (from 17 to 32 cells to >129 cells, Figure 4C). According to clonal analysis theory (reviewed elsewhere), this suggests that the pool of ancestral cells giving rise to the VCS is constant at each generation over 3 to 4 cell divisions.

The observation of large mixed and small unmixed conductive clusters suggests that outgrowth follows lineage restriction during VCS development. Both mixed and unmixed conductive clusters were observed in all compartments of the VCS, central (His, LBB and RBB), and peripheral (LPF, RPF; Table 1), suggesting that a common biphasic mode of development is shared by all components of the VCS.

Right/Left Properties of the Peripheral Conduction System

Analysis of the properties of mixed clusters in the peripheral VCS reveals a striking difference in cluster size between the right and left ventricles. As shown in Figure 5A and 5B, right PF clusters contain a small number of cells localized at the subendocardial surface of the right ventricle whereas left PF clusters contain a larger number of cells extending deeper into the ventricular wall. The numbers of conductive (GFP-
positive) and working (GFP-negative) cells in mixed PF clusters were scored by immunohistochemistry (Figure 5C). The mean number of conductive cells was found to be relatively small and similar for RPF (11 cells), LPF (9 cells), and central (12 cells) clusters (Figure 5C). In contrast, the mean number of working myocytes in mixed clusters was much higher in clusters in the left than right ventricle (RPF, 36 cells; LPF, 137 cells; P<0.05; Figure 5C) and the percentage of conductive cells correspondingly lower in LPF than RPF mixed clusters (Figure 5D). Mixed clusters represent the progeny of ancestral cells of the VCS which give rise to conductive and working myocytes. These observations suggest that, although outgrowth follows lineage restriction in all compartments of the VCS, the mechanisms by which ancestral cells give rise to conductive and working myocytes differ in the right and left ventricles. Mixed clusters in the central VCS have an even higher percentage of conductive cells than RPF clusters (Figure 5D). Thus, the cell history of ancestral cells appears to diverge between different components of the VCS.

**Prospective Cre Fate-Mapping Analysis of VCS Development Using a novel Cx40 Allele**

A prospective genetic analysis was carried out to complement the nlaacZ based retrospective analysis of VCS development. Transgenic mice were generated expressing a tamoxifen inducible Cre recombinase gene under the transcriptional control of the Cx40 locus. The expression of the transgene was monitored by an IRES-RFP reporter gene placed downstream of CreERT2. RFP was detected in atrial cardiomycocytes and ventricular trabeculae with an indistinguishable expression pattern from the endogenous Cx40 gene (Figure 6A and Online Figure I). These mice were crossed with mice carrying a conditionally activated R26R lacZ reporter gene to follow the descendants of Cx40 expressing cells during development. Tamoxifen was injected at different time points between E10.5 and E18.5, and double transgenic Cx40Cre+/R26R hearts were analyzed at P7. After tamoxifen injection at E10.5, E12.5 or E14.5 β-galactosidase labeling was observed in working and conductive myocytes of both the left and right ventricles (Figure 6B and Online Figure II). The number of β-galactosidase–positive cells was more extensive in the left than right ventricle (Figure 6B). This result suggests that at E10.5 and until E14.5, Cx40 is expressed in a cell population giving rise to both conductive and working myocytes. These cells are likely to include the ancestral cells of the VCS. The number of β-galactosidase–positive cells in the working compartment is progressively reduced in the left ventricle between E10.5 and E14.5, whereas it is constantly low in the right ventricle. These data are comparable with the difference of cellular composition of mixed clusters in the nlaacZ analysis between the left and right ventricles, where a greater working myocyte contribution was observed in mixed clones in the left than right ventricle. In contrast, after tamoxifen injection at E16.5 or E18.5, β-galactosidase–positive cells in both the left and right ventricles are almost entirely restricted to the VCS (Figure 6B). This result suggests that by E16.5 Cx40 expression is restricted to conductive foundor cells or their descendants, resulting in labeling of cells restricted to the VCS. These data reveal progressive restriction of myocardial fate to the VCS and support the existence of distinct developmental phases during establishment of the VCS.

**Discussion**

Retrospective analysis of clonally related clusters in the mammalian VCS using the nlaacZ system has provided novel insights into the origin and establishment of the VCS. Two classes of conductive clusters were identified, containing only conductive cells (unmixed conductive clusters) or both conductive and working cells (mixed clusters). Whereas the presence of mixed clusters demonstrates that conductive and working myocytes share a common progenitor, unmixed conductive clusters indicate that once cells are restricted to a conductive fate, they maintain their proliferative potential. Mixed and unmixed conductive clusters were observed in all components of the VCS, suggesting that outgrowth of cells following restriction to the conductive lineage occurs throughout the VCS. In the chick model, only one category of clone in the VCS was observed, containing both conductive and contractile myocytes. The absence of unmixed conductive clusters in the perivascular PF in the chick may be explained by differences in the timing of cell labeling events; retroviral infection is likely to occur before lineage restriction to the conduction system, whereas intragenic recombination of the nlaacZ cassette may occur at any developmental stage up to the time of observation. The relatively small size of the unmixed conductive clusters observed in our experiments in comparison to the mixed clusters suggests that they may result from a recombination event subsequent to the time of viral infection in the chick model.

In our analysis, all unmixed clusters, be they conductive or working, were observed to follow a proliferative mode of growth. However, unmixed conductive clusters are small and contain less than 30 cells, in contrast to unmixed working clusters, which contain up to 500 cells. This difference may be explained by a higher proliferation rate of the working myocardium or premature arrest of proliferation of the conductive cells. Differential proliferation rates between embryonic cardiomyocytes have been documented in the literature. During mouse embryonic development, proliferation is lower in the emerging trabeculae than in the compact ventricular myocardium. This pattern of slow proliferation correlates with the development of the peripheral and central conduction system. In support of proliferative arrest of conductive cells, Gourdie et al have shown that the number of perivascular PF is constant between E14 and E18 in chick clonal analyses experiments. The limited number of cells in unmixed conductive clusters strongly suggests that once lineage restriction to the conduction system occurs, founder cells undergo a maximum of 4 to 5 cell divisions up to the observation time.

Mixed conductive clusters, in contrast, tend to be large, and result from recombination events in ancestral cells at an earlier stage of VCS development. This period corresponds to that during which founder cells of the VCS arise. The constant number of mixed clusters over different clusters sizes indicates that this period lasts for 3 to 4 cell divisions.
Our nlaacZ retrospective analysis therefore suggests that the VCS develops in two phases, with limited proliferation following lineage restriction (or outgrowth following ingrowth). Genetic tracing analysis of trabecular myocardium using an inducible Cre recombinase allele at the Cx40 locus provides support for such a biphasic model of VCS development. Induction of recombination between E10.5 and E14.5 demonstrates that Cx40 is expressed at these time points in a population of cells giving rise to both conductive and working myocytes in each ventricle. In contrast, induction of recombination at E16.5 reveals that by this fetal stage Cx40 expression is largely restricted to the VCS lineage and that descendants contribute only to conductive myocytes.

Analyses of mixed clusters in the peripheral VCS show a striking right/left difference in the proportion of conductive versus working cardiomyocytes per cluster. Although the number of conductive cells is equivalent in both ventricles, the number of working cardiomyocytes is greater in mixed clusters of the left compared to the right ventricle. This right/left difference can be attributed to the mode of development of the ancestral cells giving rise to these mixed clusters and suggests that the ancestral cell population in the right ventricle is smaller than that in the left ventricle or is present for a shorter time period. Similarly, the pool of ancestral cells of central components of the VCS appears to be smaller than that of the peripheral VCS. However, our Cx40-Cre lineage data reveal no difference in the timing of lineage restriction to PF between the left and right ventricles, suggesting that the principle differential parameter is the number of ancestral cells. This number may in turn depend on differential parameters such as proliferation rates or divergent inductive signaling events. Left and right ventricular cells may also react differently to instructive signals because of their embryological origin in distinct progenitor cell populations, the first and second heart fields.

Strikingly the right/left difference of the mixed clusters correlates with the more extensive contribution of Cx40-derived cells to the working myocardium in the left compared to the right ventricle. These data strongly suggest that Cx40-expressing cells identify the ancestral cell population of
the VCS in the embryonic heart. Purkinje fibers are thought to develop from trabeculae that emerge from the ventricular wall from E9.5.\textsuperscript{27} Cx40 is specifically expressed in trabeculae during cardiac development, but its expression is restricted to the entire VCS in the adult heart.\textsuperscript{28} During embryonic development expression of Cx40, and of the GFP and RFP reporter genes documented here, is more extensive in the left ventricle with a gradient of expression from trabeculae to compact myocardium, in contrast to the right ventricle where expression is restricted to the trabeculae.\textsuperscript{29} The slow proliferative properties of trabeculae may explain the limited size of the working component of mixed clusters in the right ventricle compared to the left ventricle. The high proportion of conductive cells in mixed clusters in the central conduction system may similarly result from slow proliferative properties of a progenitor cell population. The existence of such a population has been proposed by others using a Tbx2-Cre lineage analysis in which a population of progenitors in the AVC giving rise to the AVN is distinguishable at early stages of development.\textsuperscript{30} Similarly, a slow proliferative population of Tbx3-expressing cells has been identified at the crest of the septum and correlated with the development of the His bundle.\textsuperscript{12}

Inductive signals coming from endothelial cells such as endothelin or neuregulin in the chick and mouse, respectively, or from epicardially derived cells, have been shown to play a role in Purkinje fiber differentiation.\textsuperscript{6,31,32} In parallel, inductive signals from the NCC have been proposed to regulate the development and maturation of the central conduction system.\textsuperscript{33,34} Our Cx40-Cre lineage study suggests that VCS lineage restriction occurs by E16.5 in both ventricles, indicating that temporal as well as spatial regulation of inductive signals may be involved in the transition to lineage restriction and the control of VCS outgrowth. Indeed, previous work has revealed a late role for Nkx2.5 in maturation of Purkinje fiber network.\textsuperscript{35}

In conclusion, retrospective lineage analysis and genetic tracing experiments have provided new insights into previous controversies existing in the literature concerning the mode of development of the VCS. We reveal that development of the mammalian VCS is biphasic: conductive myocytes develop from common progenitors with working myocytes followed by limited proliferative outgrowth. Detailed understanding of the process of VCS formation is an important issue given that ventricular arrhythmias are a major cause of morbidity and mortality and can arise from defects in establishment of the VCS during embryonic development.

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Disclosures

None.

References

Novelty and Significance

What Is Known?

- In avians, the ventricular conduction system (VCS) develops by recruitment of cardiomyocytes after an inductive signal from endothelial cells and differentiation into nonproliferative conductive cells.
- In the mouse, the central VCS develops from a population of progenitor cells distinguished by specific markers.
- Controversies exist between recruitment and outgrowth theories.

What New Information Does This Article Contribute?

- Retrospective and prospective genetic lineage analysis is applied to the origin of the mammalian VCS.
- Two phases of development underlie establishment of the VCS, lineage restriction followed by limited outgrowth.
- The mammalian Purkinje fiber network develops from connexin40-positive trabecular cells that become restricted to the conductive lineage during fetal development.

The VCS is composed of the His bundle, right and left bundle branches, and Purkinje fiber network. It represents a population of specialized cardiomyocytes ensuring rapid propagation of electric activity to coordinate ventricular contraction. Defects in VCS development contribute to cardiac arrhythmia, yet little is known about how the mammalian VCS is established. Alternative recruitment and outgrowth models have been proposed. Here, we have analyzed clonally related myocytes in the mouse heart to establish the relationship between the VCS and working cardiomyocytes. In addition, a prospective genetic fate mapping approach has been used to follow the destiny of connexin40-positive trabecular cells. Our results demonstrate that the development of the mammalian VCS is biphasic: conductive myocytes develop from ancestral connexin40-expressing cells that also give rise to working myocardium followed by limited proliferative outgrowth. Thus, in the mammalian heart both models apply, in sequence, outgrowth following recruitment or lineage restriction. Detailed understanding of the process of VCS formation is an important issue given that ventricular arrhythmias are a major cause of morbidity and mortality and can arise from defects in the establishment of the VCS during embryonic development.
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Material and Methods

Production and analysis of β-gal+ clones
The α-cardiac actin nlaacz/+ and Cx40 eGFP/+ mouse lines used in this study were genotyped as previously reported 1, 2. Double transgenic knock-in α-cardiac actin nlaacz/+;Cx40 eGFP/+ males were crossed with CD1 females to generate nlaacZ+/-;Cx40 GFP/+ mice. Hearts from three week old mice were fixed in 4% paraformaldehyde for 4 hours at 4°C, cut longitudinally in half (in a frontal plane, exposing all four chambers) to visualize the endocardial surface of the ventricles and stained with X-gal solution for 2 hours at 37°C as described 1. The number and the position of β-galactosidase positive cells within or close to the ventricular conduction system visualized by GFP fluorescence were recorded using a Zeiss Lumar stereomicroscope equipped with UV-fluorescence. Selected hearts were post-fixed in 4% paraformaldehyde and processed for sectioning and immunostaining.

Immunostaining
Hearts carrying subendocardial β-galactosidase positive clusters were transferred through graded sucrose solutions (15% and 30% w/v in PBS) before embedding in Optimal Compound Temperature (Tissue-Tek) and freezing. 10µm cryostat sections were obtained for each block and stained with anti-GFP (Molecular probes, 1/1000), anti-RFP antibodies (Rockland, 1/200), anti-actinin (Sigma, 1/500) or anti-β-galactosidase (Kappel, 1/500). For immunohistochemistry, sections were washed in PBS and incubated with primary antibody in saturation buffer (PBS 1X, 0.05% saponin, 2% BSA) overnight at 4°C. After washing, slides were incubated with biotin conjugated anti-rabbit secondary antibody (1/200) in saturation buffer and processed with peroxydase vectastain using the ABC kit (Vector) following the supplier’s instructions. Sections were mounted with aquamount (Sigma) and observed under an Axiophot microscope. For immunofluorescence, secondary antibody coupled to 488 or Cy3 were incubated in the same saturation buffer and sections were mounted with fluoromount-G and observed under an Apotome Zeiss microscope.

Optical projection tomography
Three week-old mice were anesthetised, and perfused through the dorsal aortae with prewarmed PBS to remove blood cells. Hearts were dissected and atria were removed before fixation with 4% paraformaldehyde at 4°C overnight. After washes, the apical part of the heart was sectioned to maximise antibody penetration. Hearts were incubated with a fluorescent-conjugated anti-GFP antibody (Alexa-fluor 594nm conjugate, Chemicon) for two weeks in saturation buffer (PBS, 3% BSA and 0.1% triton X-100) and washed 5 times for one hour in saturation buffer. Optical Projection Tomography was performed as described by Sharpe at al. (2002) 3. Briefly, hearts were embedded in 1% low melting agarose (LMP Agarose, Invitrogen), mounted on stainless-steel stubs, dehydrated in methanol, and cleared using benzyl alcohol-benzobenzoate (1:2). Specimens were mounted on a rotating motor and visualised using a Leica FLIII microscope fitted with GFP2 and Rhodamine filter sets. Analysis and visualisation of OPT data were performed with OsiriX Imaging software (http://www.osirix-viewer.com/). Endothelial specific GFP-staining corresponding to coronary arteries was masked with Photoshop software.

Statistical analysis
The intragenic recombination of nlaacZ into nlacZ is a random event and the frequency of N independent recombination events can be calculated by the fluctuation test of Luria and Delbruck 4.

\[ N_0 \left(\ln(Ne/N_0)\right)^{\gamma/(N!)} \]

where N0 is the number of observed hearts without cluster in the VCS (N=0) and Ne the total number of hearts analysed.

Generation and analysis of a Cx40 Cre allele
Cx40-CreERT2 mice will be described in detail elsewhere (Beyer et al. in preparation). A Cre-ERT2-IRESmRFP1 cassette has been inserted at the Cx40 locus by homologous recombination using standard techniques. 4OH-Tamoxifen (H7904, Sigma) diluted in Ethanol/Cremophor/PBS (5/45/50)
was injected intraperitoneally into pregnant females carrying the Cx40-CreERT2 cassette and the R26R reporter gene at different timepoints. Hearts were dissected at P7 and analyzed for β-galactosidase and RFP expression using the protocol described above for the detection of β-galactosidase and GFP expression.

Legend for Online Movie I:

Online Movie I. This film presents a 3D reconstruction of the ventricular conduction system (VCS) from a three-week old Cx40<sup>GFP</sup><sup>/+</sup> mouse heart obtained using optical projection tomography. The structure of the heart is identified in grey and the VCS in green representing GFP expression. The orientation of the heart is indicated by arrows: the green arrow indicates left, the red arrow ventral and the blue arrow the cardiac apex.

As the heart rotates, the different components of the VCS can be seen. The His bundle and bundle branches are localized at the crest of the interventricular septum. In the left ventricle, the Purkinje fibers form a network descending to the apex of the heart; note that Purkinje fibers are not present on the free ventricular wall between the two papillary muscles. In the right ventricle, a large Purkinje fiber network is observed on the endocardial surface of the right free wall.
Online Figure I: Endogenous Cx40, GFP and RFP expression in E16.5 mouse hearts.
(A) In situ hybridization of an E16.5 mouse heart using a Cx40 antisense riboprobe reveals Cx40 transcript accumulation in atrial cardiomyocytes and ventricular trabeculae. RV: Right ventricle. LV: Left ventricle. (B, C) Immunohistochemistry showing GFP and RFP expression in E16.5 Cx40GFP/+ (B) and Cx40Cre/+ (C) hearts. The distribution of both reporter genes is identical and indistinguishable from that of endogenous Cx40 transcripts. Arrowheads indicate Cx40 and reporter gene expression in coronary vessels.
Online Figure II: RFP and β-galactosidase-positive cells express α-actinin, a cardiomyocyte marker. Immunofluorescence on cryosections of a P7 Cx40CreET2/R26R heart after Tamoxifen injection at E14.5 shows coexpression of a cardiomyocyte marker, α-actinin (green) and RFP or β-galactosidase (red) in both ventricles. RFP identifies cells actively expressing Cx40 and β-galactosidase cells that actively or previously expressed Cx40. Expression of RFP and β-galactosidase are also observed in coronary vessels indicated by white arrows.