Epicardium-Derived Cells in Development of Annulus Fibrosis and Persistence of Accessory Pathways

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Background—The developmental mechanisms underlying the persistence of myocardial accessory atrioventricular pathways (APs) that bypass the annulus fibrosis are mainly unknown. In the present study, we investigated the role of epicardium-derived cells (EPDCs) in annulus fibrosis formation and the occurrence of APs.

Methods and Results—EPDC migration was mechanically inhibited by in ovo microsurgery in quail embryos. In ovo ECGs were recorded in wild-type (n=12) and EPDC-inhibited (n=12) hearts at Hamburger-Hamilton (HH) stages 38 to 42. Subsequently, in these EPDC-inhibited hearts (n=12) and in additional wild-type hearts (n=45; HH 38–42), ex ovo extracellular electrograms were recorded. Electrophysiological data were correlated with differentiation markers for cardiomyocytes (MLC2a) and fibroblasts (periostin). In ovo ECGs showed significantly shorter PR intervals in EPDC-inhibited hearts (45±10 ms) than in wild-type hearts (55±8 ms, 95% CI 50 to 60 ms, P=0.030), whereas the QRS durations were significantly longer in EPDC-inhibited hearts (29±14 versus 19±2 ms, 95% CI 18 to 21 ms, P=0.011). Furthermore, ex ovo extracellular electrograms (HH 38–42) displayed base-first ventricular activation in 44% (20/45) of wild-type hearts, whereas in all EPDC-inhibited hearts (100%, 12/12), the ventricular base was activated first (P<0.001). Small periostin- and MLC2a-positive APs were found mainly in the posteroseptal region of both wild-type and EPDC-inhibited hearts. Interestingly, in all (n=10) EPDC-inhibited hearts, additional large periostin-negative and MLC2a-positive APs were found in the right and left lateral free wall coursing through marked isolation defects in the annulus fibrosis until the last stages of embryonic development.

Conclusions—EPDCs play an important role in annulus fibrosis formation. EPDC outgrowth inhibition may result in marked defects in the fibrous annulus with persistence of large APs, which results in ventricular preexcitation on ECG. These APs may provide a substrate for postnatally persistent reentrant arrhythmias. (Circulation. 2008;117:1508-1517.)

Key Words: electrophysiology ■ morphogenesis ■ conduction ■ arrhythmia

Accessory atrioventricular (AV) pathway–mediated reentrant tachycardia (AVRT) is a common arrhythmia in humans.1 It is well established that these accessory pathways (APs) consist of threads of abnormal cardiac musculature that cross the fibrofatty AV grooves.2 The AP in itself, however, is an enigma, because the causative mechanisms underlying the appearance of these AV continuities remain as intriguing as they are unexplained.

It has long been thought that tissues of the endocardial AV cushions and epicardial AV grooves play a key role in the development of the electrically inert annulus fibrosis, thereby creating the isolating barrier between the atrial and ventricular tissues necessary for normal sequential activation of the heart.3,4 Recently, it was suggested that bone morphogenetic protein signaling5 and periostin-induced AV junctional myocardial remodeling6–8 also play a critical role in configuration of the isolating annulus.

It is not uncommon for annulus fibrosis formation to be incomplete at birth, and consequently, APs can be found in embryonic wild-type quail hearts at near-hatching stages of embryonic development despite proper His-Purkinje system conduction and concurrent annulus fibrosis maturation.9 These APs can persist for some time and provide the anatomic...
substrate for neonatal AVRTs, which usually resolve spontaneously during the first year of life.8,9 The reasons that AVRTs persist into childhood or adult life are not fully understood, nor have the cell types or instructive signaling routes responsible for normal annulus fibrosis formation been elucidated fully.

Epicardium-derived cells (EPDCs) migrating through the developing AV dissociated border may be crucial for proper annulus fibrosis formation, because the spatiotemporal expression of procollagen I, a marker for collagen type I synthesis, closely resembles the migratory patterns of EPDCs; whereas abundant expression of periostin at the AV junction appears to be spatiotemporally colocalized with these cells.7,10 Moreover, periostin recently was found to colocalize and directly interact with collagen type I in murine skin and heart valves.13

EPDCs originate from the proepicardium, which in avians initially develops as an outgrowth of the ventral wall of the intraembryonic splanchnopleural coelomic epithelium that covers the sinus venosus.14 After 3 days of incubation (Hamburger-Hamilton[15] [HH] stage 16 to 18), the proepicardium transforms into a cauliflower-like cluster of vesicles (in avians, generally referred to as the proepicardial organ [PEO]); which enables proepicardial cells to migrate over the myocardium to form the pericardium and epicardial monolayer.14 A population of EPDCs is subsequently generated in the subepicardium that results from epicardium-to-mesenchymal transformation.16,17 Most EPDCs are generated in the intersegmental grooves,10,17–20 subsequently migrating through the continuous AV junctional myocardium to populate the endocardium-derived AV cushions.10–12

We hypothesize that EPDCs have an inductive role in annulus fibrosis formation, which suggests that postnatal APs may persist when EPDC migration is inhibited. In wild-type and in ovo PEO-outgrowth–inhibited quail embryos at post-septated stages of embryonic development, AV conduction was studied and correlated with annulus fibrosis morphology.

Methods

Experimental Preparations

Animal experiments were approved by the Committee on Animal Welfare of the Leiden University and conducted in compliance with the “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 85-23, revised 1996). Fertilized eggs of the Japanese quail (Coturnix coturnix japonica, Leiden University) were incubated blunt end up at 37.5°C (80% humidity). Embryos were staged according to the HH criteria.15

Outgrowth of the proepicardium was inhibited by performing in ovo microsurgery, as described by Männer.21 In brief, on the third day of incubation (HH 15–18), a small piece of eggshell membrane is inserted between the dorsal wall of the heart and the pericardial vili, cranially anchored in the sinusial sulcus and caudally constrained by the coelomic wall (Figure 1).

In Ovo ECG Recordings

After termination of incubation at the desired developmental stages (HH 38–42), a subset of quail eggs (EPDC inhibition n = 12; wild-type n = 12) were prepared for in ovo ECG recording. The ECGs were recorded digitally (Prucka Engineering Inc, Houston, Tex) on a continuous basis for 10 to 15 minutes in a small, custom-built, shielded incubator (37±0.1°C). In ovo ECGs were evaluated by 2 independent observers. After completion of ECG recordings, euthanization by decapitation, and subsequent staging,

Figure 1. Representative example of mechanical EPDC-inhibition technique in an HH 16 quail embryo. A piece of eggshell membrane (EM) is placed between the right ventricle (RV) and the PEO. Nile blue staining was used to visualize transparent structures. SAS indicates sinusial sulcus; CW, coelomic wall; Dao, dorsal aorta; and A, atria.

the embryonic hearts were isolated and prepared for ex ovo extracellular electrogram recordings.

Ex Ovo Extracellular Electrogram Recordings

Extracellular electrograms were recorded at HH 38–42 in 45 wild-type and 12 EPDC-inhibited hearts during superfusion with oxygenated Tyrode solution, as described previously.8 Definitions, immunohistochemistry, morphometry, and statistical analysis are described in detail in the online-only Data Supplement.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

In Ovo ECG Recordings

The heart rate (RR interval) was similar in wild-type (n = 12) and EPDC-inhibited (n = 12) hearts (RR 246±29 versus 252±39 bpm, P = 0.648). In 3 (25%) of 12 EPDC-inhibited hearts (group B2) ECGs showed overt ventricular preexcitation reflected by (1) short nonisoelectric PR intervals, (2) initial slurring (delta wave), and (3) resultant lengthening of the QRS complexes. In these EPDC-inhibited hearts, the PR interval during sinus rhythm was significantly shorter (33±12 versus 55±8 ms, P = 0.004), whereas QRS intervals were significantly longer (50±9 versus 19±2 ms, P = 0.004) than in wild-type hearts. EPDC-inhibited hearts in group B1 (n = 9) demonstrated slightly shortened PR intervals (50±6 versus 55±8 ms, P = 0.165) and lengthened QRS intervals (22±3 versus 19±2 ms, P = 0.064) compared with wild-type hearts. In EPDC-inhibited hearts, the PR and QRS intervals were negatively correlated (Spearman’s ρ = −0.314, P = 0.320).

The ECG evaluations of 2 independent observers were in agreement: Probability values of 0.830 (PR), 0.344 (RR), and 0.187 (QRS) indicated no significant difference between the observers. Representative examples of in ovo ECG recordings in a wild-type HH 40 heart (group A), an EPDC-inhibited HH 40 heart (group B1), and an HH 41 EPDC-
inhibited heart with overt preexcitation (group B1) are shown in Figure 2A, 2B, and 2C, respectively. Tables 1 and 2 summarize the general electrophysiological (in ovo and ex ovo) characteristics of all analyzed quail hearts.

Ex Ovo Extracellular Electrogram Recordings

The AV interval in EPDC-inhibited hearts was significantly shorter than that in wild-type hearts ($62\pm11006^12 \text{versus} 79\pm26 \text{ms}$, $P=0.033$), whereas the RR interval did not differ ($125\pm35 \text{versus} 111\pm17 \text{bpm}$, $P=0.693$). In line with previous data,8 at these late stages of embryonic heart development, the ventricular base was activated prematurely in a considerable number of embryonic wild-type hearts (20/45, 44%). In contrast, all EPDC-inhibited hearts (12/12, 100%) showed earliest ventricular activation at the ventricular base. In the majority of hearts with premature ventricular base activation, the right ventricular base (RVB) was the location of first ventricular activation in both wild-type hearts (RVB = 10/20 [50%] versus left ventricular base [LVB] = 7/20 [35%]) and EPDC-inhibited hearts (RVB = 9/12 [75%] versus LVB = 3/12 [25%]). Interestingly, the interval between ventricular base and ventricular apex activation was significantly longer in EPDC-inhibited hearts ($12\pm11006^11 \text{ms}$) than in wild-type hearts showing premature ventricular base activation ($2\pm2 \text{ms}$, $P=0.001$). Representative examples of ex ovo extracellular recordings in a wild-type HH 40 heart (group C) are shown in Figure 3A and 3B, and recordings obtained in an EPDC-inhibited HH 40 heart (group D) are shown in Figure 3C and 3D.

Morphology of the Annulus Fibrosis

Macroscopically, EPDC-inhibited embryos and their hearts were consistently smaller than wild-type hearts. Furthermore, various known characteristics of the loss-of-PEO-function phenotype were observed to occur coincidently in these

Table 1. Electrophysiological (In Ovo ECG) Characteristics of Wild-Type and EPDC-Inhibited Hearts

<table>
<thead>
<tr>
<th>Group, HH Stage</th>
<th>n</th>
<th>RR, bpm</th>
<th>PR, ms</th>
<th>QRS, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (wild type)</td>
<td>12</td>
<td>246±29 (210–287)*</td>
<td>55±8 (46–74)$#$$#$$</td>
<td>19±2 (17–26)$#$$#$$</td>
</tr>
<tr>
<td>HH 38</td>
<td>3</td>
<td>214±4 (210–217)</td>
<td>50±2 (48–52)</td>
<td>18</td>
</tr>
<tr>
<td>HH 40</td>
<td>1</td>
<td>271</td>
<td>46</td>
<td>19</td>
</tr>
<tr>
<td>HH 41</td>
<td>4</td>
<td>255±27 (222–287)</td>
<td>55±5 (51–62)</td>
<td>18±1 (17–19)</td>
</tr>
<tr>
<td>HH 42</td>
<td>4</td>
<td>254±30 (212–280)</td>
<td>61±10 (50–74)</td>
<td>22±3 (20–26)</td>
</tr>
<tr>
<td>Group B (EPDC inhibition)</td>
<td>12</td>
<td>252±39 (212–333)*</td>
<td>45±10 (20–57)$\dagger\dagger$</td>
<td>29±14 (15–60)$\dagger\dagger$</td>
</tr>
<tr>
<td>Group B1</td>
<td>9</td>
<td>257±43 (213–333)</td>
<td>50±6 (41–57)$\dagger$</td>
<td>22±3 (15–25)$#$</td>
</tr>
<tr>
<td>HH 38</td>
<td>2</td>
<td>213</td>
<td>52±1 (51–52)</td>
<td>21±4 (18–23)</td>
</tr>
<tr>
<td>HH 39</td>
<td>2</td>
<td>231±1 (230–232)</td>
<td>51±9 (44–57)</td>
<td>20±7 (15–25)</td>
</tr>
<tr>
<td>HH 40</td>
<td>2</td>
<td>308±35 (283–333)</td>
<td>44±4 (41–46)</td>
<td>23±3 (21–25)</td>
</tr>
<tr>
<td>HH 41</td>
<td>2</td>
<td>263±46 (231–296)</td>
<td>50±6 (46–54)</td>
<td>22</td>
</tr>
<tr>
<td>HH 42</td>
<td>1</td>
<td>282</td>
<td>55</td>
<td>25</td>
</tr>
<tr>
<td>Group B2 (overt preexcitation)</td>
<td>3</td>
<td>238±23 (212–257)</td>
<td>33±12 (20–40)$##$</td>
<td>50±9 (42–60)$\dagger\dagger$</td>
</tr>
<tr>
<td>HH 40</td>
<td>1</td>
<td>256</td>
<td>39</td>
<td>42</td>
</tr>
<tr>
<td>HH 41</td>
<td>2</td>
<td>229±24 (212–246)</td>
<td>30±14 (20–40)</td>
<td>54±9 (47–60)</td>
</tr>
</tbody>
</table>

* $P=0.648$ (Student $t$ test); † $P=0.030$ (Mann–Whitney $U$ test); ‡ $P=0.011$ (Mann–Whitney $U$ test); § $P=0.002$; ¶ Spearman’s $r=0.790$, $P=0.002$; $\#\#=0.314$, $P=0.320$; $\dagger\dagger=0.165$ (Mann–Whitney $U$ test); $\#\#\#=0.064$ (Mann–Whitney $U$ test); $\#\#\#\#=0.004$ (Mann–Whitney $U$ test); $\dagger\dagger\dagger=0.004$ (Mann–Whitney $U$ test).
hearts (Table 3), ie, double-outlet right ventricle with ventricular septal defects (2/10), AV valve abnormalities (1/10), great artery abnormalities (1/10), coronary pathology (2/10), and myocardial hypoplasia (2/10).10,12,20–26 The central conduction axis of the EPDC-inhibited hearts did not show any histological abnormalities.

In both wild-type (n = 10) and EPDC-inhibited hearts (n = 10), small MLC2a-positive APs with comparable volumes (1.30 ± 0.40 × 10^6 μm^3 versus 1.26 ± 0.52 × 10^6 μm^3, P = 0.864) were found primarily in the posteroseptal and midseptal region of all hearts. In wild-type hearts, additional smaller (0.38 ± 0.13 × 10^6 μm^3) APs were found in the right or left lateral wall of 6 of 10 hearts (Table 3). Interestingly, however, in all EPDC-inhibited hearts, multiple MLC2a-positive APs in both the right and left lateral free-wall regions with larger volumes than the small lateral APs in wild-type hearts were found (3.14 ± 2.25 × 10^6 μm^3 versus 0.38 ± 0.13 × 10^6 μm^3; P = 0.001; Table 3; Figures 4 and 5).

As expected, temporal analysis showed a decrease in AP volume with increasing developmental stage in wild-type hearts (Pearson’s r = −0.908, P = 0.033). Maturation of the peristin-positive annulus fibrosis in EPDC-inhibited hearts, however, remained impeded compared with wild-type hearts until near-hatching stages of development.

**Peristin Expression at the Isolating AV Ring**

Peristin staining was found at the regions where EPDCs are known to be present, for example, in the endocardial AV cushions, in the atrial and ventricular subendocardium, and at variable expression levels all around the circumference of the AV ring region in both wild-type and EPDC-inhibited hearts. In both wild-type and EPDC-inhibited hearts, peristin expression was slightly lower in the right than the left AV ring region.

The small, septal, and MLC2a-positive APs in both wild-type and EPDC-inhibited hearts and the small, lateral, MLC2a-positive APs in wild-type hearts stained positive for peristin. Peristin staining on the annulus fibrosis, however, was interrupted locally at locations where broad lateral APs crossed the annulus in EPDC-inhibited hearts. In Figure 4, representative examples of MLC2a- and peristin-positive staining in the annulus fibrosis region of wild-type and EPDC-inhibited hearts at HH 39 and HH 41 are given.

**Comparison of Immunohistochemical and Electrophysiological Data**

In line with our previous report,6 AP location in wild-type postseptated hearts could not be correlated directly with ex ovo electrophysiological data; right- or left-sided APs were found both in hearts that displayed earliest ventricular activation at the RVB or LVB and in hearts with a concurrent or apex-to-base ventricular activation pattern.

In EPDC-inhibited hearts (n = 10), however, right-sided APs corresponded with premature activation of the RVB, and left-sided APs corresponded with premature activation of the LVB in 8 of 10 cases. In cases of multiple APs, the location
of earliest ventricular activation was found to correlate with
the morphologically broadest AP present (Table 3). More-
over, conduction velocity along the AP (PR interval) showed
negative correlation with the AP volume in EPDC-inhibited
hearts (Pearson’s $r = -0.696$, $P = 0.037$). The observed addi-
tional structural defects in the EPDC-inhibited hearts did not
correlate with the degree of ventricular preexcitation (in ovo
PR interval; Spearman’s $\rho = 0.000$). Interestingly, hearts in
group B$_2$ demonstrated none to only mild additional structural
abnormalities (Table 3).

**Discussion**

The key finding of the present study is that EPDCs are
essential for normal annulus fibrosis formation. Conse-
quently, inhibition of EPDC migration during cardiogenesis
may result in marked defects in the isolating annulus fibrosis
with persistence of broad accessory myocardial AV connec-
tions, which results in ventricular preexcitation.

**Embryonic Development of the Isolating Annulus
Fibrosis: The Role of EPDCs at the AV Junction**

During development of the electrically inert annulus fibrosis,
the primitive slow-conducting continuous AV junctional
myocardium of the looped embryonic heart makes way for
conduction through the AV node/His-Purkinje system, which
eventually constitutes the sole AV-conducting pathway of the
adult heart.$^{3,27}$ It is well established that AV junctional
myocardium is incorporated within the atrial myocardium by
fusion of the endocardial AV cushions and the epicardial AV
sulcus,$^{3,4}$ although state-of-the-art studies in the literature
postulate additional roles for bone morphogenetic protein
signaling and peristin in annulus fibrosis formation.$^{5–8}$

Interestingly, expression of peristin mRNA increases signif-
ically in response to mechanically regulated bone morpho-
genetic protein signaling in mesenchymal cells in culture.$^{28}$

Although the contribution of multipotent EPDCs to the heart,
both as interstitial fibroblasts (as smooth muscle cells and
fibroblasts of the coronary arteries and as mesenchymal cells in
the developing AV cushions) and in their role in formation of the
compact and trabecular myocardium and in Purkinje fiber
differentiation, has previously been shown to be indispens-
able,$^{10,23–25,29–31}$ the role of EPDCs in annulus fibrosis develop-
ment has not been studied in detail until now. The present data
show that in EPDC-inhibited hearts at late postseptated stages of
development (HH 38–42), large APs coursing through defects

![Figure 3](http://circ.ahajournals.org/)

**Figure 3.** A, Ex ovo extracellular electrograms recorded in a wild-type HH 40 heart (group C) with premature LVB activation (AV interval
78 ms). Left atrial (LA) interval (pacing artifact to LA activation) is 20 ms. B, Magnification showing LVB activation 6 ms before left ven-
tricular apex (LVA) activation. C, Recordings in an EPDC-inhibited HH 40 heart (group D) demonstrating premature LVB activation (AV
interval 67 ms). D, Magnification showing LVB activation 15 ms before LVA activation.
Table 3. Structural Abnormalities, Locations of APs, Cumulative/Individual AP Volumes, and Ventricular Activation Sequences in Wild-Type and EPDC-Inhibited Hearts

<table>
<thead>
<tr>
<th>Embryo #</th>
<th>HH Stage</th>
<th>Structural Abnormalities</th>
<th>AP Location</th>
<th>Cumulative (and Individual) AP Volumes in $10^6$ $\mu m^3$</th>
<th>Ventricular Activation Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (group A, n=10)†</td>
<td></td>
<td></td>
<td></td>
<td>1.67±0.69*</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>38</td>
<td></td>
<td>S+LL+RL</td>
<td>2.44 (1.46 + 0.56 + 0.43)</td>
<td>LVA first</td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td></td>
<td>S+LL+RL</td>
<td>2.15 (1.29 + 0.47 + 0.39)</td>
<td>LVA first</td>
</tr>
<tr>
<td>3</td>
<td>39</td>
<td></td>
<td>S+LL+RL</td>
<td>2.79 (2.10 + 0.43 + 0.26)</td>
<td>LVA first</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td></td>
<td>LL</td>
<td>1.76 (1.33 + 0.43)</td>
<td>LVB first</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
<td></td>
<td>S+LL+RL</td>
<td>1.84 (1.50 + 0.17 + 0.17)</td>
<td>LVB first</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td></td>
<td>S</td>
<td>1.50</td>
<td>LVA first</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td></td>
<td>S+RL</td>
<td>1.72 (1.24 + 0.47)</td>
<td>LVA first</td>
</tr>
<tr>
<td>8</td>
<td>41</td>
<td></td>
<td>S</td>
<td>0.77</td>
<td>LVA first</td>
</tr>
<tr>
<td>9</td>
<td>41</td>
<td></td>
<td>S</td>
<td>0.69</td>
<td>LVA first</td>
</tr>
<tr>
<td>10</td>
<td>42</td>
<td></td>
<td>S</td>
<td>1.07</td>
<td>RVB first</td>
</tr>
<tr>
<td>EPDC inhibited (group B, n=10)†</td>
<td></td>
<td></td>
<td></td>
<td>6.90±3.26*</td>
<td></td>
</tr>
<tr>
<td>Group B₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>38</td>
<td>DORV+VSD+CA</td>
<td>S+LL+RL</td>
<td>10.85 (1.29 + 4.12 + 5.45)</td>
<td>RVB first</td>
</tr>
<tr>
<td>12</td>
<td>38</td>
<td></td>
<td>S+LL+RL</td>
<td>3.22 (0.77 + 1.29 + 1.16)</td>
<td>RVB first</td>
</tr>
<tr>
<td>13</td>
<td>38</td>
<td></td>
<td>S+LL</td>
<td>4.33 (1.97 + 2.36)</td>
<td>LVB first</td>
</tr>
<tr>
<td>14</td>
<td>39</td>
<td>AVA+GAA</td>
<td>S+LL+RL</td>
<td>8.19 (1.89 + 2.49 + 3.82)</td>
<td>LVB first</td>
</tr>
<tr>
<td>15</td>
<td>39</td>
<td>DORV+VSD</td>
<td>S+LL+RL</td>
<td>7.20 (1.29 + 0.3 + 5.62)</td>
<td>RVB first</td>
</tr>
<tr>
<td>16</td>
<td>40</td>
<td>MH+CA</td>
<td>S+LL+RL</td>
<td>6.13 (0.82 + 0.56 + 4.76)</td>
<td>RVB first</td>
</tr>
<tr>
<td>17</td>
<td>41</td>
<td>MH</td>
<td>S+LL</td>
<td>2.36 (0.82 + 1.54)</td>
<td>LVB first</td>
</tr>
<tr>
<td>Group B₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>40</td>
<td></td>
<td>S+LL+RL</td>
<td>6.43 (0.52 + 1.12 + 4.80)</td>
<td>RVB first</td>
</tr>
<tr>
<td>19</td>
<td>41</td>
<td>AVA</td>
<td>S+LL+RL</td>
<td>12.95 (1.42 + 2.32 + 9.22)</td>
<td>RVB first</td>
</tr>
<tr>
<td>20</td>
<td>40</td>
<td></td>
<td>S+LL+RL</td>
<td>7.33 (1.80 + 2.57 + 2.96)</td>
<td>RVB first</td>
</tr>
</tbody>
</table>

S indicates septal; LL, left lateral; RL, right lateral; LVA, left ventricular apex; DORV, double-outlet right ventricle; VSD, ventricular septal defect; CA, coronary abnormalities; AVA, AV valve abnormalities; GAA, great artery abnormalities; and MH, myocardial hypoplasia.

Individual volumes are given parenthetically. Bold type indicates largest AP.

*Volume measurements according to the Cavalieri method.26
†P<0.001 (Student t test).

in the annulus fibrosis can be found in the right and left lateral free-wall region, although as previously described in wild-type postseptated quail hearts, additional small APs can be found, mainly in the posterosetal regions.

After mechanical EPDC inhibition (the present study), epicardial outgrowth is delayed by the insertion of a piece of eggshell membrane to block the normal cell transfer.11,20,21 Ultimately, regenerating PEO cells growing around the eggshell membrane together with pericardial mesothelial cells originating from the pharyngeal arch of the heart form a compensatory epicardium and partially rescue the normal phenotype, thereby yielding embryos with a delayed formation of PEO-derived tissues.12,16,22,23,32 Normally, migration of subepicardial EPDCs through the continuous AV junctional myocardium to the endocardial cushions of the 4-chambered heart starts from HH 32 onward.10–12,17–19 The impeded development of the AV sulcus in EPDC-inhibited hearts with consequent persistence of functional large APs at late postseptated developmental stages (HH 38–42) strongly underscores the importance of EPDCs for normal development of the annulus fibrosis. A schematic overview of the proposed role of EPDCs in normal annulus fibrosis formation is depicted in Figure 6.

Periostin Expression in the Developing Anulus Fibrosis of Wild-Type Versus EPDC-Inhibited Postseptated Hearts: Relevance for EPDC Functioning at the AV Junction

Periostin is a profibrogenic extracellular matrix protein secreted by the mesenchyme and is strongly expressed in collagen-rich fibrous connective tissues that are subjected to constant mechanical stress in vivo.6,7,33–39 It is thought that this fasciclin-I–related protein is an inhibitor of the myocardial phenotype under both physiological and pathological conditions.3,31,34–36,40,41 Moreover, periostin is likely also essential in maintaining the integrity of the fibrous heart skeleton of the mature heart.30 Multiple cellular mechanisms regulated by periostin might support destabilization of the cardiac myocyte phenotype and the formation and maintainability of the fibrous scaffold, because periostin is known to bind to fibronectin, tenascin-C, collagen V, and periostin itself.32 The spatiotemporal co-localization of periostin and EPDCs at the AV junction of the developing heart substantiates its
importance for EPDC functioning.7,10 Interestingly, periostin was recently found to interact directly with collagen type I,13 whereas the migratory patterns of EPDCs in the AV sulcus were previously established to resemble the spatiotemporal expression of procollagen I, a marker for collagen type I synthesis.10–12 The presence of periostin mRNA in the completely epicardium-derived subepicardial mesenchyme further denotes the EPDC as an important player in the dynamic interplay between molecular cues and biomechanical determinants in the AV junctional myocardium.6,7,10,11

We recently postulated that periostin expression in persistent small and mostly posteroseptal-located myocardial APs in wild-type postseptated quail hearts indicates their ultimate perinatal fate as fibrous tissue of the annulus fibrosis.8 In the present study, periostin expression in the annulus fibrosis region of EPDC-inhibited postseptated quail hearts was found to be locally interrupted at sites where large myocardial APs in the lateral free-wall region crossed the isolating annulus, which further substantiates the importance of periostin in EPDC functioning and annulus fibrosis formation. Interestingly, similar annulus fibrosis malformations occur in the periostin-knockout mouse and in mice with a conditional deletion of the Alk3 gene and consequent downregulation of periostin in the AV region.5,31,43,44

As shown in Figure 4, in both wild-type and EPDC-inhibited hearts, periostin expression was found in the endocardial AV cushions,1 of the regions where EPDCs are normally known to be present.10–12 Expression of periostin in the EPDC-deprived

Figure 4. A, Small MLC2a-positive right posteroseptal AP in an HH 39 wild-type heart. Bar=300 μm. B, Magnification of boxed area. Bar=50 μm. C, Periostin staining. Bar=50 μm. D, Periostin staining (blue) from adjacent section, superimposed on the MLC2a-stained section, showing marked periostin expression in the myocardial AP. Bar=50 μm. E, Right annulus fibrosis region of an HH 39 wild-type heart. Bar=300 μm. F, Magnification showing complete AV isolation. Bar=2 μm. G, Periostin positivity of the fibrous annulus. Bar=2 μm. H, Periostin-staining superimposed on MLC2a staining. Bar=2 μm. I, Right annulus fibrosis region of an HH 39 EPDC-inhibited heart. Bar=300 μm. J, Magnification showing a broad, persistent MLC2a-positive AP in the right anterolateral AV ring region. Bar=1 μm. K, Periostin staining. Bar=1 μm. L, Periostin staining superimposed on MLC2a staining, showing periostin negativity of the myocardial AP. Bar=1 μm. M, Left annulus fibrosis region of an HH 41 wild-type heart. Bar=300 μm. N, Magnification showing complete AV isolation. Bar=1 μm. O, Periostin positivity of the fibrous annulus. Bar=1 μm. P, Periostin staining superimposed on MLC2a staining, Bar=1 μm. Q, Left annulus fibrosis region of an HH 41 EPDC-inhibited heart. Bar=300 μm. R, Magnification showing a broad, persistent MLC2a-positive AP in the left lateral AV ring region. Bar=1 μm. S, Periostin staining. Bar=1 μm. T, Periostin staining superimposed on MLC2a staining, showing periostin negativity of the broad lateral AP. Bar=1 μm. RA indicates right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; S, sulcus; Cu, cushion; TV, tricuspid valve; MV, mitral valve; IVS, interventricular septum; and Ao, aorta.
endocardial AV cushions of the EPDC-inhibited heart, however, indicates that periostin expression is not dependent solely on the physical presence of EPDCs. Periostin expression can therefore be speculated to underlie the renowned but still largely unknown role of the endocardial AV cushions in annulus fibrosis development, whereas the constant mechanical stress at the developing AV junction can also be postulated to induce expression of this profibrogenic protein.

**Annulus Fibrosis Development and Functional AP Persistence**

Normal formation of the epicardium is described to proceed from the point of attachment of the sinoventricular mesocardium (the dorsal atrial wall facing the sinus venosus) first to the dorsal parts of the atrial and the ventricular wall and subsequently to the ventral wall of the heart. By the end of HH 26, the myocardium of the looped embryonic heart is completely covered by epicardium, whereas cardiac septation and chamber formation have not yet been completed. Outgrowth of the right ventricular dorsal wall myocardium is 1 of the last processes in cardiogenesis and expands the right ventricular inflow tract, ultimately resulting in an inevitable shift of the right side of the AV canal to become positioned above the right ventricle. Spatially, EPDC population of the right posterior annulus fibrosis region can thus only be achieved after EPDC migration through the expanding dorsal right ventricular wall, which denotes temporal postnatal AP persistence in the right posteroseptal region of embryonic wild-type quail hearts as physiological perinatal remodeling of the AV junction.

Dysynchrony in the delicate interplay between EPDCs and AV junctional cells, as shown in the EPDC-inhibited quail model, results in persistence of large lateral APs. Although EPDCs derived from the compensatory epicardium ultimately do arrive at the epicardial AV sulcus of EPDC-inhibited hearts, these cells possibly encounter AV junctional cardiomyocytes already impermissive for EPDC interaction and thus miss the appropriate time window for their intended rescue of the normal cardiac phenotype.

Persistent APs in wild-type versus EPDC-inhibited postseptated quail hearts also displayed divergent electrophysiological characteristics. In line with our previous report, morphologically persistent APs in the wild-type heart that give rise to premature ventricular activation could be found in a considerable number of cases. Inhibition of EPDC migration, however, resulted in persistence of large APs with a relatively high conduction velocity (short in ovo PR and ex ovo AV intervals), neither of which was observed in any of the wild-type hearts, and premature ventricular base activation in all cases.

Moreover, overt ventricular preexcitation was observed in a subgroup of in ovo ECGs in EPDC-inhibited hearts. Interestingly, these EPDC-inhibited hearts all demonstrated the 3 main clinical ECG features of ventricular preexcitation syndromes: (1) a short PR interval, (2) initial slurring of the QRS complex, known as the delta wave, and (3) a resultant prolonged QRS complex (Figure 2).

**Clinical Significance**

In children, the first episodes of AP-mediated AVRT occur before birth or in the first months of life in 60% of cases.
and resolve spontaneously in most cases before the age of 1 year, whereas recurrence of tachycardia at the age of 8 to 10 years occurs in approximately the remaining 30%. Bolstered by the normal postnatal evolutionary process of anatomic molding and shaping of the heart to facilitate adjustment to the increasing body mass and changes in vascular pressures, we previously postulated that under physiological circumstances, persistent functional APs at near-hatching stages of avian development provide the anatomic substrate for spontaneously resolving neonatal AVRTs.

However, any delay in EPDC migration in the developing heart may result in imperfect annulus fibrosis development and consequently in AP persistence. Clinically, most patients with persistent APs are not affected by additional cardiac pathology, although in some cases of ventricular preexcitation syndromes (eg, Wolff-Parkinson-White syndrome), AP persistence coincides with congenital cor vitia (for example, mitral valve anomalies). When EPDC migration is blocked directly after PEO formation, as in the present study, multiple mild to severe congenital heart defects will occur.

In humans, genetic alterations that affect EPDC functioning during development and occur after structural configuration of the heart has been completed may result in postnatal AP persistence in a structurally normal heart.

**Study Limitations**

Although we showed both functionally and morphologically that EPDCs are indispensable for proper annulus fibrosis formation, we were unable to demonstrate subsequent AP persistence in hatched or adult EPDC-inhibited quail hearts. Unfortunately, mechanical EPDC inhibition yields embryos in which the degree of epicardial outgrowth inhibition is directly related to the severity of the cardiac abnormalities and thus to embryonic lethality. Although the operated embryos that survived beyond HH 38 were typically those only mildly affected by comorbidity, EPDC-inhibited quail embryos are relatively small and appear to lack sufficient reserve to survive postnatally.

Interestingly, severe growth retardation, postnatal lethality, and dwarfism in adult life have recently been described in the peristin-null mouse.

**Conclusions**

EPDCs appear to be essential for proper formation of the isolating annulus fibrosis. Inhibition of EPDC migration during cardiogenesis may result in marked defects in the annulus fibrosis with persistence of broad APs, which functionally results in ventricular preexcitation. Although under physiological conditions, small septal APs in the wild-type heart temporarily remain functionally active, broad lateral APs in the EPDC-inhibited heart might provide a pathological substrate for postnatally persistent APs and AVRTs into childhood or adult life.

**Disclosures**

None.

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**CLINICAL PERSPECTIVE**

Atrioventricular reentrant tachycardia is a common arrhythmia in both children and adults; however, the causal mechanisms underlying the appearance of accessory pathways remain a subject of debate. During cardiogenesis, initial slow conduction over the circumferential myocardial AV continuity, which results in sequential activation of the preseptated heart, is replaced by apex-to-base conduction through the specialized AV node/His-Purkinje system in the septated heart. Concurrently, incorporation of the AV junctional myocardium in the lower atrial rim by fusion of the endocardial AV cushions and epicardial AV sulcus results in formation of the isolating annulus fibrosis. Migration of multipotent epicardium-derived cells (EPDCs) through the continuous AV junctional myocardium, ultimately reaching the endocardium-derived AV cushions, spatiotemporally correlates with annulus fibrosis formation. The AV junction has been postulated to be subject to physiological perinatal remodeling, which temporarily leaves functional small accessory pathways as anatomic substrates for spontaneously resolving neonatal AV reentrant tachycardias. Dyssynchrony in the delicate interplay between EPDCs and AV junctional cells, as shown in the EPDC-inhibited quail model in the present study, may result in marked defects in the isolating annulus fibrosis, with the persistence of large accessory pathways functionally resulting in ventricular preexcitation. We speculate that absence of EPDCs or a delay in EPDC migration results in the persistence of pathological substrates for postnatally persistent accessory pathways and AV reentrant tachycardias into childhood or adult life.
Epicardium-Derived Cells in Development of Annulus Fibrosis and Persistence of Accessory Pathways

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