PAR3 is essential for cyst-mediated epicardial development by establishing apical cortical domains

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INTRODUCTION

The organization of polarized epithelial cells is crucial for ontogeny and function of various tissues and organs in metazoans (Rodriguez-Boulan and Nelson, 1989). An epithelial cyst is one of the simplest architectures for differentiated epithelial cells (O’Brien et al., 2002). During embryonic development in mammals, epithelial cysts serve as progenitors for the morphogenesis of more complex organs, for example, blastocysts, somites, cysts of epicardial progenitor (EPP) cells and renal vesicles (Gumbiner, 1996; Manner et al., 2001). Epithelial cysts are composed of monolayers of polarized epithelial cells surrounded by or enclosing extracellular matrices (ECMs). Epithelial cells in completely developed cysts establish cell-cell junctions and the polarized compartmentation of cortical subdomains: the apical domain facing the lumen and the basolateral domain adhering to ECM (O’Brien et al., 2002). Cadherin-mediated cell-cell contacts and integrin-mediated contacts to ECM, particularly to basement membrane laminins, provide the spatial cues for epithelial cell polarity (Drubin and Nelson, 1996; Gumbiner, 1996; Li et al., 2003; Rodriguez-Boulan and Nelson, 1989). Results obtained from targeted gene disruption studies and Madin-Darby Canine Kidney (MDCK) cyst formation assay indicate that laminin-mediated signaling through integrin-linked kinase and Rac1 small GTPase are involved in epithelial cyst development (O’Brien et al., 2001; Sakai et al., 2003). However, the hierarchy of signaling pathways in the development of epithelial cysts remains to be clarified.

Accumulating experimental evidence suggests that the mammalian homologs of Caenorhabditis elegans polarity proteins, including PAR3 (PAR3D – Mouse Genome Informatics), have evolutionarily conserved functions in the establishment of cell polarity in various cell types (Macara, 2004; Ohno, 2001). PAR3 contains one self-oligomerization domain in the N terminus, three PDZ protein interaction domains and one aPKC-binding domain (Izumi et al., 1998; Mizuno et al., 2003). PAR3 forms a conserved protein complex with PAR6 and aPKC, and these three proteins are interdependent in their normal distribution in the mammalian epithelium (Joberty et al., 2000; Suzuki et al., 2001). The analyses of the polarity of cultured epithelial cells indicate that the polarized distribution of the PAR3/PAR6/aPKC complex is crucial for the development of these organisms by regulating polarity of various cell types (Macara, 2004; Ohno, 2001). Furthermore, recent evidence from an in vitro study imply the involvement of PAR3 in epithelial cell polarity (Hurd et al., 2003). These observations suggest the importance of PAR3 in mammalian development through the regulation of epithelial cell polarity. However, direct evidence that reveals the significance of PAR3 in mammalian development has not yet been obtained.

Among various epithelial cell cysts, EPP cell cysts play a crucial role in mammalian cardiac morphogenesis (Manner et al., 2001). The cardiac walls consist of three different tissue layers: the epicardium, myocardium and endocardium. In particular, epicardial development is a unique process that features two different mechanisms by which epicardial cells the envelope entire heart. EPP cells differentiate from mesenchymal cells in the septum transversum through mesenchymal-to-epithelial transition and then form the proepicardial serosa on the pericardial surface of the septum transversum (Manner et al., 2001). In the first mechanism, EPP cells migrate directly from the proepicardial serosa to the dorsal surface of the developing atrium and spread as a continuous epithelial sheet. This mechanism predominates in avian embryos.

Key Words: PAR3 (PARD3), Gene targeting, Epithelial cell polarity, Epithelial cyst, Heart development, Epicardium, Mouse.

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In the second mechanism, EPP cells bud out from the proepicardial serosa forming cell cysts. The EPP cell cysts float into the pericardial cavity to reach the myocardium and the cells spread from the cysts to form isolated patches of epicardial sheets (Sengbusch et al., 2002). This cyst-mediated mechanism predominates in mammals (Komiya et al., 1987; Kuhn and Lieberrer, 1988; Viragh and Challice, 1981). Finally, the epicardial sheets formed by both mechanisms coalesce to form a coherent epicardium. In addition, the epicardium plays two crucial roles in cardiac development: first, epicardial cells secrete soluble tropic factor(s) required for cardiomyocyte proliferation (Chen et al., 2002; Stuckmann et al., 2003); second, some epicardial cells subsequently develop into coronary vessels (Manner et al., 2001). Despite the fundamental roles of EPP cell cysts in mammalian cardiac development, the molecular mechanisms underlying EPP cell cyst formation are poorly understood. Targeted gene disruption in mice has revealed the important roles of the Wilms’ tumor 1 (WT1) tumor suppressor gene and the interaction of α4 integrin with fibronectin in the formation of EPP cell cysts: WT1 is required in the mesenchymal-to-epithelial transition of EPP cells, and the interaction of α4 integrin with fibronectin is involved in the proper EPP cell cyst formation (Moore et al., 1999; Sengbusch et al., 2002). However, any other molecules essential for EPP cell cyst formation have not been identified to date. We report here the first study, using PAR3-deficient mice, showing that PAR3 is essential for cyst-mediated epicardial development in mice. Although the EPP cell fate is determined in PAR3-deficient mice, EPP cells fail to form cysts, despite the retention of the activities of proliferation and migration to the myocardium. PAR3-deficient EPP cells show defects in the localization of PAR-6β, αPKC and ezrin to the apical domains but a normal localization of α4/β1 integrins and ZO1 to the basolateral domains and cell-cell junctions, respectively. Given the findings that cell-ECM and cell-cell interactions provide the spatial cues for epithelial cell polarity, we propose that PAR3 interprets the spatial cell polarity cues from integrins and cell-cell contacts to establish the apical cortical domains of EPP cells.

MATERIALS AND METHODS

Targeted disruption of mouse Par3 gene

In accordance with a previous report (Shibata et al., 1997), a 129/Sv mouse genomic DNA fragment of 14.2 kb, containing the third coding exon of the Par3 gene, was isolated. This was an MCI-Neo cassette, flanked by two loxP sequences, and one loxP sequence were introduced into the Nsol site in intron 2 and the Ndel site in intron 3, respectively. An MCI-DT-A cassette was attached at the NcoI site in intron 2 and the targeting vector was linearized at the NsiI site. As described previously (Shibata et al., 1997), the electroporation and G418 selection of J1 ES cells were carried out, and homologous recombinant ES cells were identified by Southern blot analysis, and injected into C57BL/6J blastocysts. Two independent chimeric mice were established from distinct ES cell clones, and the chimeric mice or their offspring were mated with CAG-cre transgenic female mice (Sakai and Miyazaki, 1997). All of the oocytes in hemizygous CAG-cre transgenic mice were established from distinct ES cell clones, and the chimeric mice or their offspring were mated with CAG-cre transgenic female mice (Sakai and Miyazaki, 1997). All of the oocytes in hemizygous CAG-cre transgenic mice were established from distinct ES cell clones, and the chimeric mice or their offspring were mated with CAG-cre transgenic female mice (Sakai and Miyazaki, 1997).

Western blotting

The head regions of embryonic day 11.5 (E11.5) embryos in a single litter were subjected to SDS-PAGE using discontinuous acrylamide gel (8%, 15%), and the PAR3 protein was detected by western blotting with C2-3AP pAb (0.5 μg/ml) as described previously (Hirose et al., 2002). The remaining parts of the embryos were subjected to Southern blot analysis.

Histology and immunostaining

Dissected embryos were fixed in Bouin’s fixative (3 hours, 4°C), dehydrated and embedded in paraffin wax. Sections were stained with Carazzi’s Hematoxylin (Muto pure chemicals), Eosin and phloxine B (Sigma), and photographed under a DMR microscope (Leica) equipped with a CCD camera (Pixera).

The paraffin sections of Bouin-fixed embryos were processed for heat-induced epitope retrieval using a DAKO target retrieval solution high pH (S3308) for anti-GATA4 mAb (1.5 μg/ml, G-4, Santa Cruz) and anti-aPKCα mAb (1 μg/ml, clone 23, BD Transduction Laboratories), or DAKO target retrieval solution (S1700) for anti-WT1 pAb (1 μg/ml, C-19, Santa Cruz), anti-Brdu mAb (0.5 μg/ml, B44, Becton Dickinson) and anti-αSMA mAb (34 ng/ml, 1A4, Zymed). Embryos fixed in paraformaldehyde-lysine-periodate fixative (2 hours, 4°C) (McLean and Nakane, 1974) were quenched in 100 mM NH₄Cl in PBS (1 hour, 4°C), processed for cryosectioning, and permeabilized and blocked with an Image-iT FX signal enhancer (Molecular Probes). The sections were blocked with 10% normal goat serum in PBS and subjected to immunohistochemistry or immunofluorescence labeling using the primary antibodies including anti-α4 integrin (5 μg/ml, 9C10, BD Pharmingen; 20 μg/ml, PS/2, Chemicon), VCAM1 (1 μg/ml, MVCAM.A429, BD Pharmingen), PECAM1 (0.5 μg/ml, MEC13.3, BD Pharmingen), PAR3 (1 μg/ml, C2-3AP, Hirose et al., 2002; 2 μg/ml, #07-330, Upstate), PAR6β (0.5 μg/ml, BC32AP, 1 μg/ml, BCR12AP) (Yamanaka et al., 2003), aPKCα/β (2.5 μg/ml, C-20, Santa Cruz), β1 integrin (2.5 μg/ml, MB1,2, Chemicon), ZO1 (2.5 μg/ml, ZO1-1A12, Zymed) and ezrin (1:500, 3C12, Sigma). Each pair of antibodies showed consistent staining for α4 integrin (9C10, PS/2), PAR3 (C2-3AP, #07-330), PAR6β (BC32AP, BCR12AP) and aPKC (clone 23, C-20). For immunohistochemistry, biotinylated secondary antibodies (2 μg/ml, anti-rat IgGs, BioSource; 1 μg/ml, anti-rabbit IgGs, Vector Laboratories) were visualized using a Vectastain Elite ABC kit (Vector Laboratories) and counterstained with Hematoxylin. For immunofluorescence labeling, the primary antibodies were visualized using biotinylated secondary antibodies (2 μg/ml, anti-rat IgGs, BioSource; 1.5 μg/ml, anti-mouse IgGs, Vector Laboratories) with fluorescein-avidin (2 μg/ml, Vector Laboratories), or using Cy3-, Cy5- (1 or 2 μg/ml, respectively, Amersham Bioscience) or Alexa647-conjugated (2.5 μg/ml, Molecular Probes) secondary antibodies. Images were captured under a BX50 fluorescence microscope (Olympus) equipped with a CCD camera (Photometrics). All images were arranged and labeled using Photoshop 5.5 (Adobe Systems).

BrdU-labeling analysis

Pregnant mice were intraperitoneally injected with BrDU (50 mg/kg) 1 hour before sacrifice. WT1- and BrdU-positive cells were detected as described above. Four to 10 sections were used to count at least 100 and 213 WT1-positive cells per embryo at E9.5 and E10.5, respectively.

Modified Boyden chamber assay

In accordance with a previous report (Sengbusch et al., 2002), the upper and bottom surfaces of TransWell filters (8 μm pores, #3422, Costar) were precoated with human plasma fibronectin (Gibco) in DMEM (10 μg/ml) for 2 hours at 37°C and the substrate in the upper compartment was removed. Proepicardial serosa explants from E9.5 embryos were placed in the upper compartments containing fibronectin-free DMEM and cultured for 24 hours at 37°C. The explants were fixed in 2% paraformaldehyde for 10 minutes; cells on the upper membrane surface were removed using a cotton swab and cells on the bottom membrane surface were stained for WT1.

RESULTS

Targeted disruption of the mouse Par3 gene resulted in midgestational embryonic lethality

To explore the function of PAR3 in epithelial morphogenesis in vivo, we generated PAR3-deficient mice by homologous recombination in embryonic stem (ES) cells followed by Cre-mediated

DEVELOPMENT
Fig. 1. Targeted disruption of mouse Par3 gene. 
(A) Schematic structures of the full-length Par3 protein (180 kDa) and a major splice variant (100 kDa) expressed in mouse embryos. The third exon (E3) encodes amino acids 75-135. Anti-Par3 antibodies were raised against amino acids 1-115 (N2-3E1) or 712-936 (C2-3AP). CR1 and PDZ indicate the conserved oligomerization and PDZ domains, respectively. (B) Restriction maps of wild-type mouse Par3 genomic locus, targeted vector, targeted allele (Par3^Neo^) and Par3 deleted allele (Par3^ΔE3^) obtained after cre-mediated excision of loxP-flanked region, including the third exon (E3, 181 bp, boxes), which encodes amino acids 75-135. The targeting vector has one loxP-flanked MC1-neo cassette in intron 2, one loxP site in intron 3 and one MC1-DT-A-negative selection marker. The loxP sequences are shown in triangles. The position of the 5′ external probe for Southern blot analysis is indicated. RV, EcoRV; Ap, Apal; Nc, NcoI; Ns, NsiI; Nd, NdeI. (C) Southern blotting of EcoRV-digested genomic DNA from wild-type (+/+) and heterozygous (+/ΔE3) and homozygous (ΔE3/ΔE3) littermates. The size of each band yielded by the 5′ external probe is indicated. (D) Southern blotting of EcoRV-digested genomic DNA from wild-type (+/+) and heterozygous (+/ΔE3) and homozygous (ΔE3/ΔE3) littermates. The 5′ external probe yields an 8.2 kb band from the wild-type allele, and a 7.2 kb band from the Par3^ΔE3^ allele. (E) The loss of the Par3 protein in embryos at E11.5 was examined by immunoblotting. Protein extracts from the heads of the embryos shown in D were probed with affinity-purified anti-Par3 antibodies (C2-3AP, N2-3E1). An asterisk indicates nonspecific bands. The CBB staining of blotted membrane is shown as a loading control.

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Table 1. Frequencies of genotypes in progeny from heterozygote intercrosses

<table>
<thead>
<tr>
<th>Stage</th>
<th>Genotype</th>
<th>ΔE3/ΔE3 embryos</th>
<th>Growth arrest</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔE3/ΔE3</td>
<td>Grossly normal</td>
<td>Mild</td>
</tr>
<tr>
<td>E9.5</td>
<td>80 (26.5%)</td>
<td>153 (50.7%)</td>
<td>69 (22.8%)</td>
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<tr>
<td>E10.5</td>
<td>47 (22.2%)</td>
<td>114 (53.8%)</td>
<td>51 (24.1%)</td>
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<td>41 (50.6%)</td>
<td>14 (17.3%)</td>
</tr>
<tr>
<td>E12.5</td>
<td>4 (26.7%)</td>
<td>5 (33.3%)</td>
<td>6 (40.0%)</td>
</tr>
<tr>
<td>E14.5</td>
<td>2 (40.0%)</td>
<td>2 (40.0%)</td>
<td>1 (20.0%)</td>
</tr>
<tr>
<td>E18.5</td>
<td>6 (42.9%)</td>
<td>8 (57.1%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Live born</td>
<td>72 (37.7%)</td>
<td>119 (62.3%)</td>
<td>0 (0.0%)</td>
</tr>
</tbody>
</table>

*aEmbryos with severe growth arrest, but they still had heartbeats.
†Only yolk sacs were obtained.
overt symptoms of a disease. However, no homozygous mutants (Par3<sup>E3/E3</sup>) were identified among pups born from heterozygote intercrosses (n=0/191). To determine the time of death of Par3<sup>E3/E3</sup> embryos, we analyzed embryos at different stages from E9.5 to E18.5 (Table 1). Par3<sup>E3/E3</sup> embryos at E9.5 were obtained at approximately the expected Mendelian ratio. Although all the Par3<sup>E3/E3</sup> embryos had heartbeats at E9.5, they showed developmental delay and growth retardation in various degrees. As development proceeds, growth defects in Par3<sup>E3/E3</sup> embryos became more severe and no living embryos were detected after E12.5. Thus, these data demonstrate that the disruption of the Par3 gene leads to a recessive embryonic lethality in the midgestational stage.

**Defective cardiac development in Par3<sup>E3/E3</sup> embryos**

Because midgestational lethality suggests a failure in early organogenesis in Par3<sup>E3/E3</sup> embryos (Copp, 1995), we focused our analysis on organogenesis from E9.5 to E10.5. We first examined whole-mount Par3<sup>E3/E3</sup> embryos at E9.5 and E10.5. Although most of the Par3<sup>E3/E3</sup> embryos had heartbeats at E9.5, two distinct types of defect were observed among Par3<sup>E3/E3</sup> embryos. About 40% of Par3<sup>E3/E3</sup> embryos showed severe growth retardation at E9.5 (Fig. 2C) and about 25% of Par3<sup>E3/E3</sup> embryos were dead at E10.5 (Table 1). The remaining 60-75% of Par3<sup>E3/E3</sup> embryos showed mild to slight growth retardation at E9.5 (Fig. 2B) and survived up to E10.5 (Table 1). Thus, we chose only Par3<sup>E3/E3</sup> embryos without severe growth retardation in the subsequent analyses. Even Par3<sup>E3/E3</sup> embryos with slight growth retardation showed deficits in the formations of hearts, prominent telencephalic vesicles and caudal embryonic regions resulting in shorter tails (Fig. 2E). In particular, Par3<sup>E3/E3</sup> embryos at E10.5 have the hypoplastic ventricles (asterisk) and atria (arrowhead) compared with their wild-type littermates (Fig. 2D,E'). Furthermore, severely affected Par3<sup>E3/E3</sup> embryos at E10.5 show enlarged atria and peripheral edemas (Fig. 2G), indicating a contractile failure of their hypoplastic hearts. However, such signs of a congestive cardiac failure were not found in wild-type littermates at E10.5 (Fig. 2F).

Although Par3<sup>E3/E3</sup> embryos developed properly organized somite pairs, their shorter tails may suggest slight defects in somitogenesis. Otherwise, Par3<sup>E3/E3</sup> embryos developed virtually normal neural tubes, placetas and erythroblasts in the blood vessels of yolk sacs and embryonic tissues (Fig. 2I; data not shown). Thus, these observations indicate that cardiac development is severely affected in Par3<sup>E3/E3</sup> embryos, which can mainly cause midgestational embryonic lethality.

**Epicardial development was selectively affected in Par3<sup>E3/E3</sup> embryos**

To further investigate the defective cardiogenesis in Par3<sup>E3/E3</sup> embryos, we performed histopathological analyses of the hearts of Par3<sup>E3/E3</sup> embryos at E9.5-E11.5. The cardiac walls consist of three different tissue layers: the epicardium, myocardium and endocardium (Fig. 3A). In particular, epicardial development is unique, because it is accomplished involving two different cell groups that originate from the same primordial tissue (Manner et al., 2001). EPP cells differentiate on the pericardial surface of the septum transversum to form the proepicardial serosa (Fig. 3A, ps). In the first mechanism, EPP cells directly migrate onto the atrial surface from the proepicardial serosa forming cell cysts, which float to reach the myocardial surface (Fig. 3A, red arrow).

The histopathological analysis of Par3<sup>E3/E3</sup> embryos showed no obvious defects in the formation of the heart chambers, outflow tracts (Fig. 3D,F), endocardial cushions and trabeculations in the ventricles (Fig. S1 in the supplementary material). In wild-type
PAR3 is essential for epicardial development

To assess whether epicardial development is selectively affected in Par3\textsuperscript{AE/\textAE} hearts, we confirmed the expression of specific markers of each cardiac tissue at E9.5 (Fig. 4A-E,A'-E'). \(\alpha 4\) integrin is a marker of EPP and epicardial cells (Sengbusch et al., 2002). GATA4 is essential for the formation of the proepicardium and regulates cardiogenesis (Watt et al., 2004). In wild-type littermates, the heart surface was covered with an \(\alpha 4\) integrin-positive single-layer cell sheet (Fig. 4A). In addition, the proepicardial serosa and typical EPP cell cysts budding out from them were also positive for \(\alpha 4\) integrin and GATA4 (Fig. 4A,B, arrowheads). In Par3\textsuperscript{AE/\textAE} embryos, we detected \(\alpha 4\) integrin- or GATA-4-positive cells in the proepicardial serosa (Fig. 4A',B', arrowheads), although these cells had an aberrant morphology with no cyst formation, suggesting that the epicardial cell fate is determined but cyst formation is affected. In Par3\textsuperscript{AE/\textAE} embryos, moreover, the heart surface lacked \(\alpha 4\) integrin-positive cells, indicating that epicardial development does not proceed normally. By contrast, wild-type littermates and Par3\textsuperscript{AE/\textAE} embryos showed no significant difference in the expression pattern of both markers of the myocardium (VCAM1, Fig. 4C,C'; \(\alpha 4\)SMA, Fig. 4D,D') (Kwee et al., 1995; Ruzicka and Schwartz, 1988) and endocardium (PECAM1; Fig. 4E,F) (Vecchi et al., 1994). In addition, the expression pattern of GATA4 in the myocardium and endocardium was not affected in Par3\textsuperscript{AE/\textAE} embryos (Fig. 4B'). Furthermore, the proliferation of myocardial and endocardial cells was not affected in Par3\textsuperscript{AE/\textAE} embryos at E9.5 and E10.5 (Fig. S2 in the supplementary material). Taken together, despite the retention of the initial determination of the EPP cell fate, further epicardial development is selectively affected in Par3\textsuperscript{AE/\textAE} embryos.

To further examine the role of PAR3 in epicardial development, we analyzed the expression of the PAR3 protein in the hearts of embryos at E9.5. In wild-type hearts (Fig. 4F), the EPP (arrows) and epicardial cells (arrowheads) expressed both \(\alpha 4\) integrin and PAR3. PAR3 labeled with ZO1 and PECAM1 (arrows). These observations suggest that PAR3 concentrates in the cell-cell junctions of the EPP and epicardial cells similarly to other epithelial cells (Hirose et al., 2002; Izumi et al., 1998; Manabe et al., 2002; Takaki et al., 2001). However, Par3\textsuperscript{AE/\textAE} hearts showed no detectable signals for PAR3 (Fig. 4F',G'). In addition, an excessively large number of \(\alpha 4\) integrin-positive EPP cells accumulated in the Par3\textsuperscript{AE/\textAE} proepicardial serosa at E9.5-E10.5 (Fig. 3C). In wild-type hearts (Fig. 3D), the myocardial serosa and proepicardial serosa at E9.5-E10.5 (Fig. 3C) were not detected at E9.5-E10.5 and the myocardium was not covered with a single layer of flat cells at E10.5-11.5 (Fig. 3E, arrowheads). In further epicardial development is selectively affected in Par3\textsuperscript{AE/\textAE} embryos.
serosa (Fig. 4F), suggesting a defect of EPP cells in budding out from the proepicardial serosa. Thus, these observations are consistent with that of a selective defect in epicardial development in Par3$^{A3/E3}$ embryos.

**EPP cell cyst formation, not migration and proliferation of epicardial progenitor cells, was affected in Par3$^{A3/E3}$ embryos**

As indicated in Fig. 3A, EPP cells reach the heart via two mechanisms. To investigate the function of PAR3 in epicardial development further, the sections of paraffin-embedded embryos at E10.5 were stained for WT1, another marker of EPP and epicardial cells (Moore et al., 1999). In wild-type hearts (Fig. 5A), typical EPP cell cysts (arrow) were identified and confirmed positive for WT1. Although no EPP cell cysts were identified in Par3$^{A3/E3}$ embryos, WT1-positive cells were identified in the proepicardial serosa (Fig. 5B). In addition, we found WT1-positive cell layers continuing directly from the proepicardial serosa and partially covering the atria of Par3$^{A3/E3}$ embryos, as observed in wild-type littermates (Fig. 5A,B, arrowheads). We next assessed the proliferation activity of EPP cells by BrdU pulse labeling at E9.5 and E10.5. There was no significant difference in the proportion of BrdU-labeled EPP cells expressing WT1 between Par3$^{A3/E3}$ and control littermates (Fig. 5C,D), suggesting that the proliferation of Par3$^{A3/E3}$ EPP cells is not affected. Thus, although Par3$^{A3/E3}$ EPP cells fail to form typical EPP cell cysts, they appear to retain the activities of proliferation and migration to the myocardium.

To confirm the notion that Par3$^{A3/E3}$ EPP cells retain migration activity, this activity was evaluated by a modified Boyden chamber assay (Fig. 5E-G) (Sengbusch et al., 2002). There was no significant
difference in the number of migrated EPP cells between wild-type and Par3 ΔE3/E3 EPP cells (Fig. 5G). Taken together with the observation of WT1-positive cells in Par3 ΔE3/E3 embryos, these observations suggest that PAR3 is responsible for the cyst-mediated, but not direct migration-mediated, development of the epicardium.

**The organization of apical cortical domains was disturbed in Par3 ΔE3/E3 EPP cells**

EPP cell cyst formation is selectively affected in Par3 ΔE3/E3 embryos. It is crucial for the development of coherent epithelial cell cysts to establish cell-cell junctions and the polarized compartmentation of cortical subdomains; the apical domain facing the lumen and the basolateral domain adhering to ECM (O’Brien et al., 2002). To further analyze the etiology of defective EPP cell cyst formation in Par3 ΔE3/E3 embryos, we assessed the subcellular localizations of the markers of cortical subdomains and cell-cell junctions. First, we examined the apical protein localization. In wild-type EPP cells, PAR6β and aPKC showed an apical cortical association as observed in other epithelial cells (Fig. 6A, B, E, G, white arrowheads) (Verstovsek et al., 1998; Yamanaka et al., 2003). In Par3 ΔE3/E3 EPP cells, however, both PAR-6β and aPKC were generally mislocalized into the cytoplasm (Fig. 6C, D, F, H, white arrowheads). Furthermore, the apical cortical localization of ezrin in wild-type EPP cells (Saotome et al., 2004) was hardly observed in Par3 ΔE3/E3 EPP cells (Fig. 6I, J, arrowheads). By contrast, both wild-type and Par3 ΔE3/E3 EPP cells at E10.5 showed the same short linear or punctate pattern of ZO1 staining at cell-cell contact sites (Fig. 6E-H, black arrowheads) (Stevenson et al., 1986), indicating the establishment of cell-cell junctions in both wild-type and Par3 ΔE3/E3 EPP cells as shown previously (Komiyama et al., 1987; Viragh and Challice, 1981). Last, we investigated the basal protein localization. Both α4 and β1 integrins localized to the basal domains of both wild-type and Par3 ΔE3/E3 EPP cells (Fig. 6E-J, arrows). The margins of the basal domains were positive for ZO1 in both wild-type and Par3 ΔE3/E3 EPP cells (Fig. 6E-H, black arrowheads). Thus, PAR3 is crucial for the apical cortical localization of PAR6β, aPKC and ezrin, but is dispensable for the basal localization of α4/β1 integrins and the cell-cell localization of ZO1. Taken together, we conclude that PAR3 plays an essential role in cyst-mediated epicardial development by establishing apical cortical domains.

**DISCUSSION**

Cell cysts composed of polarized epithelial cells play fundamental roles in embryonic development in mammals. For epithelial cell polarization, cell-cell contacts and integrin-mediated contacts to ECM provide spatial cues (Drubin and Nelson, 1996; Li et al., 2003; Rodriguez-Boulan and Nelson, 1989). The precise molecular mechanisms that interpret these cues for the establishment of polarized apical and basolateral plasma membrane domains remain unclarified. The PAR3/aPKC/PAR6 complex is involved in the regulation of the plasma membrane domain compartmentation of one-cell zygotes in C. elegans, neuronal and epithelial cells in Drosophila embryos, and cultured mammalian cells (Macara, 2004; Ohno, 2001). We provide here the first evidence that PAR3 plays crucial roles in the development of mouse embryos by establishing epithelial cell polarity. Our results suggest that PAR3 is essential for the establishment of apical cortical domains, but is dispensable for that of basolateral domains and the formation of cell-cell junctions in EPP cells.

A deficiency in PAR3 in mice leads to a defective cardiac development and the following observations support that this defect can cause midgestational embryonic lethality. First, the
histopathological and immunohistological studies showed that Par3ΔE3ΔE3 embryos lack the epicardium (Figs 3, 4). Second, epicardial cells secrete soluble tropic factor(s) required for cardiomyocyte proliferation (Chen et al., 2002; Stockmann et al., 2003). Consistently, Par3ΔE3ΔE3 hearts at E10.5 are hypoplastic (Fig. 2) and Par3ΔE3ΔE3 embryos at E11.5 have thin atrial walls (see Fig. S1 in the supplementary material) as observed in other mutant mice showing defective epicardial development (Kwee et al., 1995; Moore et al., 1999; Watt et al., 2004). Then what is the ontogeny of defective epicardial development in Par3ΔE3ΔE3 embryos? Our observations suggest that EPP cell cyst formation is defective in Par3ΔE3ΔE3 embryos. The EPP cell cysts are responsible for epicardial development in mammals (Manner et al., 2001). Although EPP cells were detected in Par3ΔE3ΔE3 embryos and proliferated normally, EPP cell cysts were not detected in Par3ΔE3ΔE3 embryos (Figs 3-5). The accumulation of an excessively large number of EPP cells in the proepicardial serosa in Par3ΔE3ΔE3 embryos may underlie the defect of EPP cells in forming cysts and budding out from the proepicardial serosa (Fig. 4). Furthermore, the disturbance in the polarity of Par3ΔE3ΔE3 EPP cells strongly suggests a defective development of EPP cell cysts (Fig. 6), because the organization of epithelial cell polarity is crucial for cyst formation (O’Briен et al., 2002). By contrast, the attachment of EPP cells to the heart would not be affected in Par3ΔE3ΔE3 embryos, because the interaction between α4 integrin and VCAM1 is sufficient for the attachment of EPP cell cysts to the heart surface (Kwee et al., 1995; Sengbusch et al., 2002) and both proteins are expressed in the developing epicardium (Moore et al., 1999; Takaki et al., 2001), we found that not all of these cells are affected in Par3ΔE3ΔE3 hearts (Fig. 3). Indeed, we confirmed that Par3ΔE3ΔE3 EPP cells retain the α4 integrin-dependent attachment and migration activities in vivo and in vitro (Fig. 5). Nonetheless, all of the Par3ΔE3ΔE3 embryos fail to develop the epicardium and suffer from cardiac dysfunctions.

Although PAR3 is expressed in all of the epithelial cells investigated (Hirose et al., 2002; Izumi et al., 1998; Manabe et al., 2002; Takaki et al., 2001), we found that not all of these cells are affected in Par3ΔE3ΔE3 embryos. One possible explanation is the functional redundancy by the PAR3-related protein PAR3L/PAR3β (Gao et al., 2002; Kohjima et al., 2002). It has been shown that PAR3L/PAR3β colocalizes with ZO1 to cell-cell junctions (Kohjima et al., 2002) and in vitro translated PAR3L/PAR3β can associate with PAR6β in the same way as PAR3 can (Gao et al., 2002). Indeed, we found the normal localization of PAR6β in various Par3ΔE3ΔE3 epithelial cells, which are not affected morphologically (data not shown). By contrast, the development of the EPP cell cysts and mesonephric vesicles is affected in Par3ΔE3ΔE3 embryos with a mislocalization of PAR6β (Fig. 6; see Fig. S3 in the supplementary content).
material). Taken together with the observation that mammalian PAR6 is required for epithelial cell polarity in vitro (Macara, 2004; Ohno, 2001), these observations raise a possibility that among various epithelial tissues, the polarity of EPP cell cysts and mesonephric vesicles depends on PAR3 to localize PAR6β to the apical cortical domains.

Although we did not observe an apparent mislocalization of ZO1 in cell-cell junctions in the Par3A/Par6E3 epithelium, we found defects in the morphogenesis of epithelial tissues that originate from mesenchymal tissues via mesenchymal-to-epithelial transition: that is, EPP cell cysts and mesonephric vesicles (see Fig. S3 in the supplementary material). The morphogenesis of these two tissues requires a dynamic regulation of epithelial cell polarity and a remodeling of cell-cell and cell-ECM interactions. During a dynamic epithelial cell polarization in vitro, ZO1 localizes to immature cell-cell junctions, whereas PAR3 is recruited into the junctional complex later than ZO1 (Suzuki et al., 2002). Accumulating evidence from studies using cultured epithelial cells indicates that the PAR3/Par6/aPKC complex is required in epithelial cell polarization by regulating the maturation of junctional structures (Hirose et al., 2002; Suzuki et al., 2001; Yamanaka et al., 2003). Our results are consistent with these observations and imply that PAR3 is essential for the dynamic remodeling of cell-cell junctions that leads to the establishment of epithelial cell polarity of EPP cell cysts and mesonephric vesicles.

Our results indicate that PAR3 is crucial for EPP cell cyst formation in the proepicardial serosa by regulating plasma membrane polarity. We also found that mesonephric vesicle formation is defective in Par3A/Par6E3 embryos with a multilayered epithelium and disorganized lumens (see Fig. S3 in the supplementary material). Considering the finding that the accomplishment of epithelial cell polarization requires multiple steps starting from cell-cell and cell-ECM contacts (Drubin and Nelson, 1996; Gumbiner, 1996; Li et al., 2003; Rodriguez-Boulan and Nelson, 1989), we propose a simple model in which PAR3 interprets polarity cues provided by cell-cell and cell-ECM interactions leading to the establishment of apical cortical domains (Fig. 7). This model is based on the following observations. First, the expression of WT1, GATA4 and α4 integrin is retained in Par3A/Par6E3 EPP cells (Figs 4, 5), indicating PAR3 is dispensable for mesenchymal-to-epithelial transition that specifies the EPP cell fate. Second, the normal localization of ZO1, α4 integrin and β1 integrin in Par3A/Par6E3 EPP cells suggest that the cell-cell and cell-ECM interactions can provide polarity cues without PAR3 (Fig. 6). Furthermore, the α4 integrin-dependent cell migration activity is retained in Par3A/Par6E3 EPP cells (Fig. 5) (Sengbusch et al., 2002), indicating that signals through α4 integrin is functional in Par3A/Par6E3 EPP cells. Last, PAR3 plays a crucial role in the localization of PAR6β, aPKC and ezrin to the apical cortical domains of EPP cells (Fig. 6). Ezrin performs an essential function in organizing the apical cortical domains of the developing small intestinal epithelium in mice (Saotome et al., 2004). In addition, several lines of evidence imply that it is crucial for the formation of MDCK epithelial cysts to regulate epithelial cell polarity via the PAR3/aPKC/PAR6 complex and its downstream CRB3/PALS1/PATJ complex in the apical cortical domains (Hurd et al., 2003; Lemmers et al., 2004; Roh et al., 2003). Our model is further supported by the observation that par3 RNAi in C. elegans disturbs the apical organization of distal spermathecal cells (Aono et al., 2004). Taken together, our present data suggest that PAR3 is responsible for epithelial cyst formations by interpreting polarity cues from the cell-cell and cell-ECM interactions that lead to the establishment of apical cortical domains.

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Supplementary material
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