p27Kip1 in cell-cell adhesion and cell polarity
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Cell polarity development and protein trafficking in mutant hepatocytes lacking E-cadherin/β-catenin-based adherens junctions

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Polarization in AJ cells

Abstract

Using a mutant hepatocyte cell line in which E-cadherin and β-catenin are completely depleted from the cell surface and, consequently, fail to form adherens junctions, we have investigated adherens junction requirement for apical-basolateral polarity development and polarized membrane trafficking. It is shown that these hepatocytes retain the capacity to form functional tight junctions, develop full apical-basolateral cell polarity, and assemble a subapical cortical F-actin network, although with a noted delay and a striking defect in subsequent apical lumen remodeling. Interestingly, whereas hepatocytes typically target the type II plasma membrane protein dipeptidyl peptidase IV first to the basolateral surface, followed by its transcytosis to the apical domain, hepatocytes lacking E-cadherin-based adherens junctions target dipeptidyl peptidase IV directly to the apical surface. Basolateral surface-directed transport of other proteins or lipids was not visibly affected in hepatocytes lacking E-cadherin-based adherens junctions. Together, our data show that E-cadherin/β-catenin-based adherens junctions are dispensable for tight junction formation and apical lumen biogenesis, but not for apical lumen remodeling, and, in addition, suggest a requirement for E-cadherin/β-catenin-based adherens junctions with regard to the indirect apical trafficking of specific proteins in hepatocytes.
Introduction

Epithelial cells are characterized by their asymmetric cell surface organization, which includes an apical domain that faces the lumen, a basolateral domain facing the underlying tissue, and a lateral domain facing neighboring cells. Cell surface asymmetry, coupled to the polarized distribution of intracellular organelles and cytoskeleton, as well as to cellular processes such as proliferation, is crucial for epithelial functioning. Perturbed or loss of epithelial asymmetry is a hallmark of many epithelial diseases including carcinogenesis. The process by which epithelial cells develop apical-basolateral surface asymmetry is still poorly understood (Le Bivic et al., 2005). Nectin- and E-cadherin-mediated cell-cell adhesion is believed to serve as an initial cue for the development of apical-basolateral cell polarity by first defining the lateral plasma membrane. Studies in cultured epithelial cells (Vega-Salas et al., 1987, McNeill et al., 1990; Miyoshi and Takai, 2005) and Drosophila (Tepass & Hartenstein 1994) suggest that E-cadherin induces the formation of a primordial junction complex that subsequently matures (i.e. forms a multiprotein complex) and eventually evolves into distinct and spatially separated adherens junctions (AJs) and tight junctions (TJs; Miyoshi and Takai, 2005). TJs then allow for the spatial segregation of apical and basolateral surface-enriched proteins and lipids within the plasma membrane bilayer by acting as a physical barrier, and the separation of the extracellular apical and basolateral milieus by acting as a diffusion barrier (van Meer & Simons 1986). It is believed that the formation of TJs generally requires prior formation and maintenance of AJs (Miyoshi and Takai, 2005, and references herein).

In addition to a role for E-cadherin in the assembly of AJs and TJs, E-cadherin-mediated cell-cell adhesion and the subsequent remodeling of the actin and microtubule cytoskeleton have been proposed to give rise to basolateral but not apical targeting patches for intracellular trafficking pathways originating
from the Golgi apparatus (Yeaman et al., 1999; 2004). This is examplified by autosomal dominant polycystic kidney disease (ADPKD), characterized by perturbations in the polarized phenotype and function of epithelial cells, in which mutated polycystin supposedly disrupts E-cadherin-dependent cytoarchitecture and, in this way, adversely affect protein assemblies that are crucial for basolateral but not apical trafficking (Charron et al., 2000). The recruitment of basolateral trafficking pathways to E-cadherin-mediated cell-cell adhesion sites, thus spatially segregating these from apical trafficking pathways, would then reinforce apical-basolateral cell polarity (Yeaman et al., 1999; 2004). Interestingly however, hepatocytes target some apical proteins first to the basolateral surface prior to their delivery to the apical surface via transcytosis, which may suggest that E-cadherin-mediated cell-cell adhesion is not essential for spatially diverting basolateral and apical trafficking routes. Alternatively, some apical proteins may have additional functions at the basolateral surface and therefore contain basolateral targeting signals (Weisz et al., 1992), or hepatocytes may simply lack the molecular machinery for sorting subclasses of proteins directly to the apical surface (Bastaki et al., 2002).

In this study we have investigated AJ requirement for apical-basolateral polarity development and polarized membrane trafficking, by taking advantage of a mutant hepatocyte cell line, HepG2-AJ-, in which E-cadherin and β-catenin are completely depleted from the cell surface and, consequently, fail to form AJs. It is shown that E-cadherin-mediated cell-cell adhesion is dispensable for TJ formation and apical-basolateral polarity development, but not for subsequent apical lumen remodeling. In addition, it is shown that E-cadherin-dependent cell-cell adhesion is required for the initial basolateral targeting of specific apical proteins, and that these proteins are targeted directly to the apical surface when E-cadherin-mediated cell-cell adhesion is absent. The implications of these findings with regard to our
understanding of cell polarity and polarized membrane trafficking are discussed.

**Figure 1** The HepG2-AJ’ cell line presents a defect in E-cadherin and β-catenin localization. The parental HepG2 and AJ-defective HepG2-Aj- cell lines were grown during 72hrs onto ethanol sterilized coverslips in culture medium, fixed and stained against β-catenin (A) and E-cadherin (B). Arrowheads highlight the presence of β-catenin (A) and E-cadherin (B) at the cell-cell contact in the parental HepG2 while the HepG2-AJ’ cell line display an intracellular localization of these proteins. Bar is 20µM.
Results

**HepG2-AJ-, a mutant HepG2 cell line lacking E-cadherin/β-catenin-based adherens junctions**

We have recently described a human hepatocyte HepG2 cell line that stably overexpresses a mutated p27^{Kip1} that cannot be phosphorylated on Ser-10 and displays a striking reduction in cell-cell adhesion strength when compared to parental HepG2 cells (Théard et al., chapter 3). Detailed analysis revealed that in these mutant cells, β-catenin is sequestered intracellularly, preventing it from interacting with E-cadherin which, in turn, accumulates in the endoplasmic reticulum (figure 1). Consequently, β-catenin and E-cadherin do not interact with each other and adherens junctions are not formed. It is important to note that these cells do not remain as single cells and do engage in cell-to-cell interactions that may be mediated by desmosomes and additional non-cadherin-based mechanisms. In this paper, we will refer to this cell line as HepG2-AJ.

**HepG2-AJ- cells develop intercellular apical lumens**

Parental HepG2 cells (ATCC #HB8065) display a typical hepatocellular polarity characterized by the presence of an apical surface domain, representing the bile canaliculus surface (BC), which is enclosed by adjacent cells (van IJzendoorn et al, 1997; van IJzendoorn and Hoekstra, 2000). BCs can be readily visualized by electron microscopy, and appear as intercellular spherical structures containing numerous microvilli.

We examined HepG2-AJ- cells with regard to their ability to develop apical BC surface domains. HepG2-AJ- cells were cultured as described in materials and methods for three days, fixed, and processed for microscopy. Light microscopical analysis of 1µm thick sections (figure 2A) as well as transmission electron microscopical evaluation (figure 2B) clearly demonstrates that HepG2-AJ- cells developed intercellular BCs (figure 2A, white arrow), in the absence of other noticeable forms of cell-cell...
Figure 2 The HepG2-AJ cell line displays a polarized phenotype. In 1µM thick section from embedded samples observed in light microscopy (A, Bar) as in electron microscopy (B, Bar), apical looking structures (white arrow) were observed in the HepG2-AJ cell line despite an obvious defect in cell-cell contact (cell boundaries are depicted by dashed lines). The localization of diverse well characterized apical markers were assessed in the HepG2-AJ cell line, and appeared to be correctly localized: radixin (C, left panel), 5'nucleotidase (C, middle panel) and MDR1 (C, right panel) immunostainings are depicted (lower panels) with the corresponding phase contrast (upper panels). Bar is 20µm.
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Interactions. The BCs in HepG2-AJ− cells appeared somewhat smaller when compared to their size in parental HepG2 cells (not shown) and were filled with microvilli (Figure 2B). To further confirm the apical nature of the membranes lining the BCs, we examined the localization of proteins that are known to reside in or at the bile canalicular surface in vivo. The peripheral ERM-family protein radixin, the GPI-anchored ecto-enzyme 5′-nucleotidase (5′NT), and the multi-membrane spanning ABC-transporter MDR1 all localized exclusively to the BC (Figure 2C). This was also the case for other BC-associated proteins including DPP IV and villin (see below). Note in figure 2C that groups of cells interact with each other only via their apical surface (arrows) without any other noticeable cell-cell contacts.

In order to add a quantitative measure to apical polarity in HepG2-AJ− cells, cells were cultured for 72 h, fixed, and stained with a monoclonal antibody raised against the microvilli-associated protein villin, and DAPI to visualize the nuclei. We then determined by immunofluorescence the number of BCs and cells in several microscopical fields and expressed cell polarity as the ratio BC/100 cells (van IJzendoorn and Hoekstra, 1998). 72 h after plating, HepG2-AJ− cells had developed 25.8 ± 0.5 BCs/100 cells. Given that the formation of one intercellular BC requires at least two polarized cells, at least 51.6% of the HepG2-AJ− cells thus acquired a polarized phenotype. In comparison, parental HepG2 cells developed 48.6 ± 2.0 BCs/100 cells (i.e. 97.2% of the cells acquired a polarized phenotype). Together, the data indicate that cells lacking E-cadherin/β-catenin-based AJs are capable of developing apical bile canalicular lumens, albeit to a lesser extent.

Apical lumens appear intracellularly prior to their exposure at the extracellular space in HepG2-AJ− cells

Evidently, the formation of an intercellular apical lumen requires the participation of at least two cells and, hence, cell-cell interactions. When examining cell polarity under phase contrast, it was readily observed that microvilli-lined BC displayed a

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Figure 3 Apical lumens appear intracellularly prior to their exposure at the extracellular space in HepG2-AJ cells. The repartition of the different types of apical structures, depicted in A (N represents the nuclei, the grey circles represent the apical structures), was evaluated after staining 2 and 3-days old cells against the apical protein villin per 100 nuclei labeled with Hoechst (C). BCs are visible at 48 and 72 hrs in HepG2 (B1, 2), while the HepG2-AJ cells display mainly “docked” structures at 48 hours (B3) but either “docked” vesicles and BC at 72hrs (B4, 5).

 transient intracellular appearance in HepG2-AJ cells at earlier time points. Upon prolonged culture, these structures appear to move vectorially to the cell surface facing adjacent cells where they “dock” and eventually merge with the plasma membrane to establish an intercellular lumen. Such intracellular BCs correspond to the vacuolar apical compartment (VAC) described
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in epithelial cells cultured in low-calcium medium (Vega-Salas et al., 1987), and typically arise in epithelial cells that are prevented from establishing cell-cell adhesion (Vega-Salas et al., 1988). In order to address the appearance of VACs and intercellular lumens in a quantitative manner as a function of time, cells were cultured for 48 or 72 h and fixed. Apical lumens were then identified by microvilli appearance under phase contrast microscopy (figure 3B) and categorized as either located intracellular, “docked” to the cell surface or intercellular (see figure 3A). In parental HepG2 cells, more than 75% and 80% of the identified apical structures were intercellular after 48 and 72 hours, respectively (figure 3C). Most of the remaining apical structures were categorized as “docked” and only few (< 10%) intracellular lumens were observed. In striking contrast, in HepG2-AJ cells, only 25% and 45% of the apical structures appeared intercellular after 48 and 72 hours of culture, respectively. 45 and 28% of the apical structures were categorized as intracellular after 48 and 72 hours, respectively, and the remainder was categorized as “docked” (Figure 3C). These data suggest that apical lumens in HepG2-AJ remain largely intracellular prior to their docking and merging with a severe delay with the plasma membrane.

HepG2-AJ cells form tight junctions with functional “barrier” and “fence” functions

To determine whether functional TJ develop in HepG2-AJ cells, the subcellular distribution of two well established TJ proteins, ZO-1 and PAR-3 was examined. In both HepG2 and HepG2-AJ cells, a typical ZO-1 and PAR-3 staining pattern, i.e. perpendicular to the basolateral-apical axis, bordering the apical plasma membrane, was observed (figure 4A and 4B). Furthermore, electron microscopical analysis revealed the presence of electron-dense surface structures bordering the apical plasma membrane domain (figure 4C). In order to verify whether these TJs were functional, we checked their ability to segregate fluids present in the BC lumen and in the basolateral medium (i.e.
“gate” or “barrier” function), and determined their ability to prevent lateral diffusion of membrane lipids between apical and basolateral surface domains (“fence” function). The “gate” or “barrier” function was first tested using 5-carboxyfluorescein diacetate (CFDA), a fluorescent substrate of apical ABC transporters which, following cellular loading and cleavage by intracellular esterases that render it membrane-impermeable, is rapidly translocated by these transporters into the apical lumen. As shown in figure 4D, CFDA was effectively retained in the apical lumen in between adjacent HepG2-AJ- cells, suggesting that functional TJs are present that prevent paracellular leakage from the apical to the basolateral compartment. HepG2-AJ- cells were also incubated with Lucifer Yellow, a water-soluble fluorescent dye commonly used to test epithelial monolayer permeability, at 4°C (to prevent endocytosis) for 30 min. As shown in figure 4E, no Lucifer Yellow could be detected in apical lumens identified by phase contrast microscopy, indicating that no paracellular transport of solute from the basolateral to the apical compartment occurred, and corroborating the above finding of the existence of an efficient TJ “barrier” function in HepG2-AJ- cells. The “fence” function of TJs was subsequently investigated using the fluorescent lipid analog C₆NBD-sphingomyelin. HepG2-AJ- cells were incubated with C₆NBD-sphingomyelin at 4°C for 30 min, which allows the probe to incorporate in the basolateral plasma membrane without being endocytosed (van IJzendoorn et al., 1997, Ait Slimane et al., 2003). Cells were then incubated for an additional 15 in buffer at 4°C. As shown in figure 4F, the lipid probe was readily observed into the basolateral plasma membrane whereas no labeling of apical plasma membranes was observed, indicating that diffusion of the lipid probe from the basolateral to the apical plasma membrane was prevented. In an additional experiment, HepG2-AJ- cells were incubated with C₆NBD-sphingomyelin at 37°C for 30 min, which allows its incorporation into the basolateral plasma membrane and subsequent transcytosis to the apical surface (van IJzendoorn et al., 1997).
Figure 4 Tight Junctions are still present and functional in the HepG2-AJ' cell line. The localization of the tight junction proteins ZO-1 (A, green) and PAR3 (B, green) was assayed in 3-days old HepG2-AJ' cells. Nuclei were stained by Hoechst (blue) and the phase contrast is shown in the upper panel. Bars are 20 µm. (C) HepG2-AJ' cells were observed in electron microscopy to detect the presence of tight junctions (arrow). Enlargement is depicted in the lower panel. TJ: tight junction, D: desmosome. Bars are 500nm for the upper panel and 60nm in the lower panel. (D) The gate function of the tight junctions was tested using CFDA, a
Fluorescent substrate of the multidrug resistant protein MRP2. The HepG2-AJ' cells were loaded 30 minutes at 37°C with 0.5µM CFDA. After extensive washes, the content of the BCs was observed under fluorescence microscopy (green). The phase contrast is depicted in the upper panel. Bar is 20µm. (E) To confirm the functionality of the gate functions of the TJ, the p27S10A cells were incubated 30 min at 4°C in presence of 100µM of Lucifer Yellow. After extensive washes, the content of the apical structures (white arrows) in fluorescence was assayed under light microscopy. Upper panel: Phase contrast; Lower panel, Lucifer yellow. Bar is 10µm. (F,G) The fence function of the tight junctions was tested using sphingomyelin-NBD. When incorporated at 4°C, the NBD-SM remains at the basolateral membrane and remains stained on time (F, 0min vs. 15min.) whereas at 37°C, followed by a back exchange as described in Materials and Methods, only the BCs is labeled and stays stained in time (G, 0min vs. 15min) without leakage of the lipids to the basolateral part of the membrane. Arrows indicate the localization of the BCs. Bars are 20µm.

Fluorescent lipids remaining in the basolateral surface were then depleted by a back exchange procedure at 4°C (see Materials and Methods), leaving the lipid probe only in the apical plasma membrane and intracellular vesicles. HepG2-AJ' cells were then incubated in buffer at 4°C for another 15 min. As shown in figure 4G, fluorescent lipids were retained at the apical surface and no fluorescent lipids were detected at the basolateral domain, suggesting that lipids do not diffuse from the apical to the basolateral domain. Taken together, these data show that HepG2-AJ' cells develop TJ's with functional “barrier” and “fence” functions.

**HepG2-AJ' cells present a defect in apical lumen remodeling**

In the liver, hepatocytes are arranged into one-to-two cell thick plates that allow formation of a tubular bile canalicular network. When parental HepG2 are cultured for 5 days or longer, intercellular BC elongate and are remodeled to form large
multicellular canalicular lumens (figure 5A). In striking contrast, long-term cultures of HepG2-AJ' cells failed to undergo these morphological changes, and BC remain as small spherical lumens in between two cells (figure 5B). These data suggest that E-cadherin/β-catenin-based AJs are dispensable for initial apical-basolateral polarity development, but are required for subsequent remodeling of BC lumens into multicellular tubes.

**Figure 5** The HepG2-AJ' presents a defect in bile canaliculi remodeling. The parental HepG2 and HepG2-AJ' cell lines were grown during 5 days onto ethanol sterilized coverslips in culture medium, fixed and stained against phalloidin-TRITC to label the actin surrounding the bile canaliculi. Nuclei are stained by Hoechst (blue). Bar is 20µm.

**Blocking E-cadherin function in parental HepG2 cells mimics the phenotype of HepG2-AJ' cells**

We next investigated cell polarity development in parental HepG2 cells when cultured in medium containing E-cadherin blocking antibodies. Culturing HepG2 cells for 48 h in the presence of E-cadherin blocking antibodies resulted in the displacement of E-cadherin from the cell surface to intracellular structures (figure 6A). The loss of E-cadherin from the cell surface, surprisingly, did not alter the subcellular localization of β-catenin, which remained at the plasma membrane, even at sites where the plasma membranes of neighboring cells did not physically interact (figure...
Regardless of the intracellular accumulation of E-cadherin, HepG2 cells cultured in the presence of E-cadherin blocking antibodies developed intercellular BC lumens (figure 6A) that were bordered by ZO-1-positive TJ's (figure 6C), and contained the apical surface associated ERM protein radixin (figure 6D). Moreover, BC were surrounded by a dense actin meshwork as in the parental HepG2 (figure 6E). These data support our findings in HepG2-AJ- cells that E-cadherin and, consequently, AJ's are dispensable for the development of apical-basolateral cell polarity.

HepG2-AJ- cells display altered apical plasma membrane-directed trafficking of dipeptidyl peptidase IV

In hepatocytes, direct trafficking pathways from the Golgi apparatus to either apical or basolateral plasma membrane domains exist (Zaal et al., 1994). These pathways are selectively employed by different apically destined proteins and lipids. Newly synthesized polytopic ABC transporters such as MDR1 and MRP2, and the copper transporter ATP7B are targeted directly to the BC membranes (Sai et al., 1999; Kipp and Arias, 2000; 2002; Ait Slimane et al., 2003). In contrast, apical resident proteins such as the glycosylphosphatidylinositol (GPI)-anchored protein 5'nucleotidase (5'NT), and the type II single transmembrane ectoenzyme dipeptidyl peptidase (DPP) IV, are first targeted to the basolateral surface, followed by their internalization and transcytosis to the BC membranes (Bartles et al., 1987; Schell et al., 1992; Ihrke et al., 1998; Bastaki et al., 2002; Ait Slimane et al., 2003). All basolateral proteins are presumed to be targeted to the basolateral surface directly (i.e. without prior delivery to the apical domain). We compared the trafficking itineraries of MDR1, 5'NT, and DPP IV between HepG2 and HepG2-AJ- cells in order to obtain information as to the possible involvement of E-cadherin-mediated cell-cell adhesion in these events. The steady state distributions of MDR1
Parental HepG2 display polarity when cultured in presence of E-cadherin Blocking Antibody. The parental HepG2 were grown during 3 days in presence of E-cadherin blocking antibody. The coverslips were then stained against E-cadherin (A) to verify the efficiency of the blocking, the AJ protein β-catenin (B) with an enlargement of the precise localization of this protein at the membrane (B, framed), the TJ protein ZO-1 (C), the apical marker radixin (D) and the actin marker phalloidin-TRITC (E) as described in Materials and Methods and depicted on the top as immunofluorescence (IF). The corresponding phase contrast pictures of these IF were depicted on the bottom. Bar is 20µm.
Figure 7 Steady state localization of dipeptidyl peptidase IV in HepG2 and in HepG2-AJ⁺ cells. 3 days-old Hep G2 cells were fixed and stained against DPPIV after permeabilisation (A, Dapi; B, DPPIV; C, merge) or not (D, Dapi; E, DPPIV; F, merge) with 0.25% triton X-100. In parallel, HepG2-AJ⁺ were labeled after permeabilisation with triton X-100 (G, Dapi; H, DPPIV; I, merge) or not (J, Dapi; K, DPPIV; L, merge). Bars are 10µm.
Figure 8 Trafficking of dipeptidyl peptidase IV in HepG2 and in HepG2-AJ- cells. HepG2 and HepG2-AJ- cells were exposed to antibodies raised against extracellular epitopes of DPPIV at 4°C for 30 min, followed by a wash and chase of the antibodies at 37°C for 60 min. A, HepG2 cells before the chase (0 min) in phase contrast. B, Immunofluorescence with DPPIV in green and
Hoechst nucleus staining in blue. C and D depict phase contrast and immunofluorescence respectively after 60 min of chase. Similarly, E and F show phase contrast (E) and immunofluorescence of DPPIV and Hoechst (F) before the chase and G and H are the corresponding pictures after 60 min of chase.

(figure 2C), 5’NT (figure 2C) in HepG2-AJ− cells revealed extensive and almost exclusive localization of the proteins at the BC. DPPIV was abundantly present at the BC (Figure 7A-C) and in the basolateral plasma membrane (figure 7D-F) of parental HepG2 cells, evidenced by immunostaining in permeabilized and non-permeabilized cells, respectively. However, in HepG2-AJ− cells, DPPIV was only detected at the BC (figure 7G-I) and not at the basolateral surface (figure 7J-L), suggesting that in these cells DPPIV was delivered to the apical domain without prior basolateral delivery. To further investigate this, the basolateral surface of living HepG2 and HepG2-AJ− cells was exposed at 4°C for 30 min to antibodies raised against extracellular epitopes on DPPIV, followed by a wash and chase of the antibodies at 37°C for 60 min. Cells were then fixed on ice and processed for immunofluorescence analyses. Basolateral staining and basolateral to apical transcytosis was readily observed in HepG2 cells (figure 8A-D), consistent with its indirect trafficking in hepatocytes (Bastaki et al., 2002). However, in striking contrast, no basolateral staining and transcytosis of DPPIV was observed in HepG2-AJ− cells (figure 8E-H) despite its abundant apical localization at steady state (figure 7G-I), suggesting that delivery of DPPIV to the apical plasma membrane in HepG2-AJ− cells occurs via a direct pathway and does not involve passage through the basolateral domain. Similar transcytosis assays were performed using antibodies raised against extracellular epitopes on MDR1 and 5’NT. In case of MDR1, we detected no basolateral staining (figure 9A) or basolateral to apical transcytosis in HepG2-AJ− cells (figure 9B), consistent with the reported direct trafficking of MDR1 from the Golgi apparatus to
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the BC in HepG2 cells (Kipp and Arias, 2000; Aït Slimane et al., 2003). In case of 5’NT, basolateral staining (figure 9C) and basolateral to apical transcytosis (figure 9D) was readily observed in HepG2-AJ- cells, similar as reported in parental HepG2 cells (Aït Slimane et al., 2003). These data suggest that E-cadherin-mediated cell-cell adhesions in hepatocytes may direct the basolateral targeting of DPPIV, and perhaps other single transmembrane proteins, but not that of other apical proteins such as GPI-anchored 5’NT. Moreover, for DPPIV these data imply that, in contrast to previous suggestions, hepatocytes do possess a Golgi-based machinery for directly sorting single transmembrane apical proteins like DPPIV.

**Figure 9** Trafficking of MDR1 and 5’NT. HepG2-AJ- cells were exposed to antibodies raised against extracellular epitopes of MDR1 (A,B) and 5’NT (C,D) at 4°C for 30 min, followed by a wash and chase of the antibodies at 37°C for 60 min. (A) MDR1, before the chase. (B) Same staining after 60 min of chase. Similarly, (C) 5’NT, before the chase. (D) Same staining after 60 min of chase. Bar is 20µm.
Discussion

In this study, we demonstrate that hepatic HepG2-AJ- cells develop functional tight junctions and apical-basolateral polarity in the absence of E-cadherin-mediated cell-cell adhesions. Earlier studies with cultured cells suggested that E-cadherin-dependent cell-cell adhesion is the founding event of epithelial polarity (reviewed in Le Bivic et al., 2005; Miyoshi and Takai, 2005, and references herein). These studies were mostly based on monitoring the ordered recruitment of specific AJ and TJ proteins in time following disruption of the epithelial monolayer by calcium deprivation. Expression of a mutant of E-cadherin that lacks only the adhesive extracellular domain, however, was shown to increase TJ assembly, and suggested that the hierarchical regulation of apical junction complexes is not absolute (Troxell et al., 2000). More recently, Baas et al., (2004) demonstrated that single intestinal epithelial cells that overexpress an inducible activator of LKB1/PAR4 develop an apical “brush border” domain with apical proteins that are segregated from basolateral proteins by TJs, indicating polarity development without the need for prior E-cadherin-based cell-cell contact establishment. Harris and Peifer (2004, 2005) demonstrated that polarity of epithelial cells in early stages of Drosophila development can be established without formation of E-cadherin complexes. Our data, obtained in a hepatic cell line that expresses endogenous wild-type E-cadherin but is incapable of establishing E-cadherin-based AJs, provide new evidence for and underscore the concept that E-cadherin function at the cell surface is dispensable for TJ formation and apical-basolateral polarity development per se.

Although apical-basolateral polarity establishment was evident, development of intercellular apical lumens in freshly plated HepG2-AJ- cell cultures was delayed, and microvilli-lined BC were observed intracellularly prior to their appearance in between adjacent cells. This is consistent with earlier reports
which showed that loss of cell-cell adhesion precludes the activation of a signaling cascade that is required for the efficient delivery of apical vesicles to the plasma membrane. Instead, apical vesicles accumulate in the cytoplasm and, by means of homotypic fusion, give rise to intracellular vacuolar apical compartments (VAC) that functionally and structurally resemble intercellular apical lumens (Vega-Salas et al., 1987; 1988; 1993). In contrast to the cells in those studies, which lacked any type of cell-cell adhesion, intracellular apical lumens in HepG2-AJ- cells evidently “dock” with the cell surface and form functional intercellular apical lumens. Conceivably, a delay in the acquisition of cell-cell adhesion strength due to the lack of functional E-cadherin is responsible for the temporary impediment of intercellular apical lumen formation in HepG2-AJ- cells.

Whereas E-cadherin-mediated cell-cell adhesion appears dispensable for formation of functional TJ and apical-basolateral polarity, it is not for subsequent apical lumen morphogenesis and remodeling. Thus, whereas prolonged culturing of parental HepG2 cells results in the development of elongated and multicellular apical lumens, this does not occur in HepG2-AJ- cell cultures, in which apical lumens remain as spherical lumens in between mostly two cells. Apical lumen remodeling in HepG2 cells is preceded by the deposition of extracellular matrix and subsequent inhibition of Rho kinase and myosin-II signaling, which are instrumental for cell multilayering and, in this way, cell-to-cell reorientation (H. Herrema, D. Czajkowska, D. Théard, D. Kalicharan, B. Zolghadr, D. Hoekstra, S. van IJzendoorn, manuscript in preparation). Probably, E-cadherin-regulated cell-cell adhesion is essential for dynamic cell-to-cell (re)orientation and apical lumen remodeling. Indeed, loss of E-cadherin prevents the development of epithelial tissues and is embryonically lethal (Johnson et al., 1986; Harris et al., 2005).

Interestingly, the indirect apical trafficking pathway of the type II transmembrane protein DPPIV in HepG2 cells changes to
a direct apical trafficking route in HepG2-AJ’ cells. In contrast, the indirect and direct trafficking itineraries of the GPI-anchored 5’NT and the polytopic ABC transporters MRP2 and MDR1/3, respectively, are similar in HepG2 and HepG2-AJ’ cells. In different epithelial cell lines, the resident apical membrane protein DPPIV can be targeted to both apical and basolateral surface but with a highly variable apical:basolateral ratio (Aït Slimane et al., 2001, and references herein). Whereas in MDCK cells most DPPIV is transported directly to the apical domain (Casanova et al., 1991), in hepatocytes DPPIV is predominantly sorted to the basolateral domain, followed by its transcytosis to the apical surface (Bastaki et al., 2002; Aït Slimane et al., 2003). Consistent with the diversity in polarized trafficking, DPPIV has been proposed to contain both apical and basolateral sorting signals (Weisz et al., 1992), the dominance of which may be a reflection of competing sorting and trafficking machineries (Zurzolo et al., 1992). In contrast to previous suggestions (Bastaki et al., 2002), our data indicate that hepatocytes do harbor the machinery for directly sorting DPPIV, and possibly other single transmembrane proteins, to the apical surface. However, the direct apical sorting of DPPIV in hepatic cells is likely to be overruled by other mechanisms that target DPPIV to the basolateral surface. Our data implicate cell surface-associated E-cadherin as an important element of such an overruling mechanism. Strikingly, the indirect trafficking of the GPI-anchored 5’NT remains unaltered like the direct trafficking of polytopic ABC transporters in HepG2-AJ’ cells, indicating that the influence of E-cadherin on polarized membrane trafficking is restricted to specific (classes of) proteins, and/or restricted to specific (basolateral surface-directed) transport pathways.

Taken together, the results reported in this study provide novel insight as to the requirement of E-cadherin at the cell surface for the establishment and subsequent remodeling of TJ and apical-basolateral surface asymmetry, and for regulating the
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targeting of specific apical proteins to spatially segregated areas of the cell surface.

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Materials and Methods

Cell culture
HepG2 cells were cultured in DMEM with 4500 mg/L glucose and supplemented with 10% FCS and antibiotics. Culture media were changed every other day. The p27S10A cell line, here named HepG2-AJ, was created as described in chapter 3 and cultured in medium supplemented with G418.

Fluorescence microscopy
Cells cultured on glass coverslips for 3 days were fixed and stained as described in van der Wouden et al. (2002). The antibodies used were: monoclonal anti-β-catenin antibody (Transduction laboratories), polyclonal anti-E-cadherin (kindly provided by M. Wheelock), monoclonal anti-MDR1 (C219; Abcam), polyclonal anti-radixin (Sigma), monoclonal anti-S’nucleotidase, monoclonal anti-DPPIV (gift from Dr. Hauri, Biozentrum der Universität Basel, Basel, Switzerland), monoclonal anti-ZO-1 (Zymed), polyclonal anti-PAR3 (Upstate) and Alexa Fluor 488 or 596 goat anti-rabbit or -mouse (Molecular Probes) as secondary antibodies, together with Hoechst 33528 (5ng/ml) to label nuclei. For the E-cadherin blocking experiments, the cells were cultured in presence of E-cadherin blocking antibody (gift from M. Wheelock, 1:50) and processed for microscopy.
For polarity determination, the cells were fixed with -20°C for 5 min and stained with monoclonal anti-villin antibodies. The level of polarity was determined counting the number of apical structures (VACs, docked or BCs) per 100 nuclei. Since a BC is shared by at least two cells, we approximated it as two apical structures to be able to compare with the number of VACs and Docked structures. Actin staining of apical structures was done as described in van der Wouden et al. (2002). Cells were examined with an Olympus Provis AX70 fluorescence microscope.

Electron Microscopy
Cells were washed several times with 6.8% saccharose to remove serum in 0.1 M cacodylate buffer, pH 7.4 at RT then fixed for 30 min at RT in 2% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4. The cells were rinsed three times in the same buffer with
6.8% sucrose and subsequently postfixed in 2% OsO₄/3% K₄Fe(CN)₆ in 0.2 M cacodylate buffer (pH 7.4) at 4°C for 1hr. After rinsing in 0.1 M cacodylate buffer, pH 7.4 and dehydration in a graded alcohol series, the cells were embedded in Epon 812 and polymerised a weekend over at 58°C. Finally, Ultrathin sections (60 nm) were cut and stained with uranyl acetate and lead citrate. The sections were examined using a Philips CM 100 electron microscope operating at 80 kV, and micrographs were taken.

**Determination of the TJ “barrier” function**
To determine whether TJ allow paracellular diffusion from the BC lumen to the basolateral medium, we incubated the cells with 5-carboxyfluorescein diacetate (CFDA, 0.5µM, Sigma), at 37°C for 30 minutes to allow its internalization and subsequent translocation into the BC lumen by the MRP2 ABC-transporter. After extensive washes, the capacity of BC to contain the fluorescent CFDA was analyzed with a fluorescent microscope.
To test whether fluids could pass from the culture medium to the BC lumen, we incubated the basolateral side of the cells with Lucifer Yellow (100µM, Sigma) on ice for 30 min and, following extensive washes on ice, determined the absence or presence of fluorescence in the BC lumens.

**Determination of the TJ “fence” function**
To check the “fence” function of TJ, we used C₆-NBD-SM as previously described (van der Wouden, 2002). Briefly, HepG2 cells were cooled to 4°C by washing with ice-cold HBSS and incubated with 4 µM C₆-NBD-SM for 30 min at 4°C to label the basolateral plasma membrane without allowing endocytosis. Following extensive washes, the cells were observed under fluorescence microscopy to detect any leakage from the basolateral stained side to the BC membrane. In an alternative experiment, cells were incubated with C₆-NBD-SM at 37°C for 30 min to allow endocytosis and subsequent transcytosis to the BC membrane. To terminate transport, the cells were cooled by washing three times with ice-cold HBSS. Fluorescent lipids remaining in at the basolateral surface was removed by a back-exchange procedure as described in van der Wouden et al., 2002). After extensive washes, the cells were observed under
fluorescence microscopy to detect any diffusion of the lipid probe from the apical to the basolateral surface.

**Transcytosis assay**

3 day-old HepG2 and HepG2-AJ- were washed, incubated in HBSS containing antibodies raised against extracellular epitopes on DPPIV (monoclonal anti-DPPIV, gift from Dr. Hauri, Basel), MDR1 (monoclonal anti-MDR1, Abcam) and 5’ nucleotidase (monoclonal anti-5’NT) at 4°C for 30 min. After extensive washes, the antibodies were chased at 37°C for 60 min. Cells were then fixed on ice and processed for immunofluorescence analyses as described above.