Distinct roles of cadherin-6 and E-cadherin in tubulogenesis and lumen formation

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ABSTRACT **Classic cadherins are important regulators of tissue morphogenesis. The predominant cadherin in epithelial cells, E-cadherin, has been extensively studied because of its critical role in normal epithelial development and carcinogenesis. Epithelial cells may also coexpress other cadherins, but their roles are less clear. The Madin Darby canine kidney (MDCK) cell line has been a popular mammalian model to investigate the role of E-cadherin in epithelial polarization and tubulogenesis. However, MDCK cells also express relatively high levels of cadherin-6, and it is unclear whether the functions of this cadherin are redundant to those of E-cadherin. We investigate the specific roles of both cadherins using a knockdown approach. Although we find that both cadherins are able to form adherens junctions at the basolateral surface, we show that they have specific and mutually exclusive roles in epithelial morphogenesis. Specifically, we find that cadherin-6 functions as an inhibitor of tubulogenesis, whereas E-cadherin is required for lumen formation. Ablation of cadherin-6 leads to the spontaneous formation of tubules, which depends on increased phosphoinositide 3-kinase (PI3K) activity. In contrast, loss of E-cadherin inhibits lumen formation by a mechanism independent of PI3K.**

Monitoring Editor
Keith E. Mostov
University of California, San Francisco

Received: Jan 14, 2011
Revised: Apr 11, 2011
Accepted: Apr 12, 2011

INTRODUCTION

Many organs, including the lung, mammary gland, and kidney, have a basic architecture of branching epithelial tubules. Activated growth factor receptor tyrosine kinases drive the dynamic rearrangements of epithelial cells required for tubulogenesis by regulating cell migration, proliferation, polarization, and adhesion (Zegers *et al.*, 2003b; Affolter *et al.*, 2009). These receptor tyrosine kinase–activated pathways act in concert with cell-matrix signaling. In particular, the loss of function of β1- and α6- or α8-integrins inhibits branching morphogenesis during development of the kidney (Wu *et al.*, 2009; Zhang *et al.*, 2009), the mammary gland (Klinowska *et al.*, 1999), the submandibular gland (Koyama *et al.*, 2009), and the lung (Benjamin *et al.*, 2009). Epithelial remodeling also crucially depends on regulation of cell–cell adhesion, but the role of cell–cell adhesion in tubulogenesis is poorly defined. Classic cadherins are transmembrane adhesion molecules that mediate adhesion of neighboring cells via calcium-dependent homotypic interactions between their ectodomains (Wheelock and Johnson, 2003). The predominant classic cadherin in all epithelial cells is E-cadherin, but dependent on the tissue, other cadherins, such as P-cadherin (Tinkle *et al.*, 2008) and cadherin-6 (Stewart *et al.*, 2000), may be coexpressed. As compared with E-cadherin, which is extensively studied, little is known about these coexpressed cadherins, although they are often assumed to have redundant functions, at least in adult tissue. Cadherins localize at the lateral membrane, where they form the foundation of the adherens junction complex. Sequential interactions of the cadherin cytoplasmic domain with β-catenin and α-catenin within adherens junctions eventually link cadherin to the actin cytoskeleton (Jou *et al.*, 1995; Harris and Tepass, 2010). The cytoplasmic domain of cadherin also interacts with an array of other regulatory proteins. Together, these cadherin-interacting proteins mediate tissue morphogenesis, and E-cadherin in particular is crucial for epithelial morphogenesis, as it controls apicobasolateral polarity and cell proliferation and survival and interacts with the force-generating actin cytoskeleton (Gumbiner, 1996; Harris and Tepass, 2010). These pivotal functions of E-cadherin are emphasized by the fact that embryos of E-cadherin knockout mice die very early in development, when the earliest (trophectodermal) epithelium forms (Larue *et al.*, 1994). Furthermore, the loss of E-cadherin

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E11-01-0038) on April 20, 2011.

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Abbreviations used: DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; EMT, epithelial to mesenchymal transition; HGF, hepatocyte growth factor; MDCK, Madin Darby canine kidney; MET; mesenchymal to epithelial transition; PI3K, phosphoinositol 3-kinase; shRNA, small hairpin RNA.

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is a hallmark of epithelial-to-mesenchymal transition (EMT), a process in which cells loose cell–cell contact, delaminate from the epithelium, and acquire a mesenchymal and migratory phenotype (Yang and Weinberg, 2008). The loss of function of E-cadherin also correlates with increased invasiveness and metastasis of tumors (Bracke *et al.*, 1996).

Madin Darby canine kidney (MDCK) cells are a classic mammalian system to analyze the assembly of E-cadherin–based adherens junctions and to study the function of E-cadherin in epithelial polarization (Vega-Salas *et al.*, 1987; McCrea *et al.*, 1991; McNeill *et al.*, 1993; Adams *et al.*, 1996; Takaishi *et al.*, 1997; Nejsum and Nelson, 2007). When grown in three-dimensional (3D) culture, they are also a well-established model for tubulogenesis (Montesano *et al.*, 1991a; Santos *et al.*, 1994; Zegers *et al.*, 2003b). Single cells embedded in an extracellular matrix such as collagen I proliferate and organize into cysts, which are spherical, hollow structures formed by monolayer of highly polarized epithelial cells surrounding a single central lumen. Treatment of cysts with hepatocyte growth factor (HGF) induces the formation of branching tubules in a process that recapitulates aspects of renal tubulogenesis (Rosario and Birchmeier, 2003; Zegers *et al.*, 2003b). Tubulogenesis in MDCK cysts is thought to involve a partial EMT (O'Brien *et al.*, 2004; Leroy and Mostov, 2007). Indeed, cells within the developing tubule transiently lose their apical-basolateral polarization (Pollack *et al.*, 1998), but unlike in EMT, they maintain E-cadherin–mediated adhesion (Pollack *et al.*, 1998; Leroy and Mostov, 2007). Cadherin-mediated cell–cell adhesion is also maintained during branching morphogenesis in vivo in the mammary and salivary glands (Ewald *et al.*, 2008; Walker *et al.*, 2008) and even required for the latter process (Walker *et al.*, 2008). In contrast, tube formation in the *Drosophila* salivary gland requires a temporal down-regulation of DE-cadherin (Pirraglia *et al.*, 2006).

To further investigate the role of cadherins in epithelial morphogenesis, we focused on cadherins that are coexpressed in MDCK cells. In addition to E-cadherin, these cells express relatively high levels of cadherin-6 (Stewart *et al.*, 2000). Cadherin-6 is mainly found in the kidney and the CNS. It is highly expressed during embryonic kidney development, where it promotes mesenchymal-to-epithelial transition (MET) (Cho *et al.*, 1998; Dahl *et al.*, 2002). Cadherin-6 is also required for nephrogenesis in zebrafish (Kubota *et al.*, 2007). Findings that endogenous levels of E-cadherin and cadherin-6 are regulated by distinct mechanisms in MDCK cells (Stewart *et al.*, 2000) suggest that they serve different functions. Despite this, little is known about the specific roles of E-cadherin and cadherin-6 in epithelial morphogenesis. This is also due to the fact that several approaches designed to inhibit E-cadherin, such as expression of dominant-negative E-cadherin or "calcium switch" experiments, equally affect cadherin-6 (Troxell *et al.*, 2001; Liu *et al.*, 2010) and E-cadherin. Recent studies in which both E-cadherin and cadherin-6 were knocked down in MDCK cells with small hairpin RNA (shRNA) suggested that E-cadherin and cadherin-6 play redundant roles in maintaining the epithelial monolayer (Capaldo and Macara, 2007; den Elzen *et al.*, 2009). Here we investigated the specific roles of E-cadherin and cadherin-6 in epithelial morphogenesis using a knockdown approach. We demonstrate that both cadherins serve distinct roles in generating and maintaining a polarized epithelial phenotype. Specifically, we find that cadherin-6, but not E-cadherin, functions to inhibit tubulogenesis. In contrast, E-cadherin is required for the formation of a single lumen, whereas cadherin-6 is dispensable for lumen formation. We furthermore show that tubulogenesis induced by ablation of cadherin-6 involves activation of phosphoinositide 3-kinase (PI3K), but that this pathway is not required for the mechanism by which E-cadherin regulates lumen formation.

RESULTS

Conditional knockdown of cadherin-6 or E-cadherin induces distinct phenotypes

To determine the roles of cadherin-6 and E-cadherin in epithelial morphogenesis in 3D cell culture, we created cell lines that conditionally express shRNA specific to cadherin-6 (cad6-KD), E-cadherin (Ecad-KD), or both (6/E-KD). We used two different targeting sequences specific for either cadherin and selected clones in which doxycycline-induced expression of the shRNAs effectively and specifically down-regulated cadherin-6 or E-cadherin protein expression by at least 95% (Figure 1A). We chose one clone for each hairpin to cadherin-6 (cad6-KD1, cad6-KD2) and E-cadherin (Ecad-KD1, Ecad-KD2) for all subsequent experiments. As conditional shRNA expression was not completely tight in all clones, we used cells that conditionally express a nontargeting shRNA as control. Western blot analysis showed that the loss of cadherin-6 was not compensated by increased protein levels of E-cadherin in any of cad6-KD clones we established. Some of the Ecad-KD clones we generated had slightly elevated (20–40%) cadherin-6 levels (unpublished data), but this was not the case for the clones we selected for our experiments (Ecad-KD1 and Ecad-KD2; Figure 1A). Loss of either cadherin isoform did not result in a loss of cell–cell adhesion as judged by an aggregation assay (compare control in Figure 1, B to C and D). In contrast, knockdown of both cadherins severely disrupted cell–cell adhesion in this assay (Figure 1E).

Single MDCK cells suspended in a collagen I matrix proliferate to form fluid-filled cysts that consist of a monolayer of polarized cells enclosing a central lumen (O'Brien *et al.*, 2002) (Figure 1F, control). Knockdown of cadherin-6 strongly altered this spherical organization and led to the spontaneous formation of large, lumen-containing tubules (Figure 1G, arrows). In addition, relatively narrow spines emanated from the surface of the cysts or tubules (Figure 1C, arrowheads). The appearance and size of these tubules increased with time (images show 13-d-old cysts). The distinct tubulogenic phenotype of the cad6-KD cysts can be appreciated from low-power projection images generated from confocal image stacks of control and cad6-KD cysts stained for nuclei and filamentous actin (Supplemental Figure 1, compare A to C). This image also shows that the phenotype is specific for the loss of cadherin-6, as re-expression of EGFP-tagged mouse cadherin-6 that had been mutated to make it refractory to the cad6 shRNA hairpin (Supplemental Figure 1, E–F″) restored the spherical phenotype (Supplemental Figure 1D). In contrast to the tubulogenic phenotype of cad6-KD, Ecad-KD cells formed spherical cysts that had no discernible lumens when analyzed by phase-contrast microscopy (Figure 1H, Supplemental Figure 1B). The viability of the 6/E-KD cells was low in 3D culture as judged by a ~10-fold decrease in cyst number as compared with controls (unpublished data). The remaining cysts exhibit a combination of phenotypes of those seen in the Ecad-KD and cad6-KD cysts, in that all of the 6/E-KD cysts had filled lumens, but many exhibited (filled) tubules as well (Figure 1, I and J). Of interest, the cad6-KD and Ecad-KD phenotypes were almost completely mutually exclusive. Thus quantification of the phenotypes as shown in Figure 1F demonstrated that 45–54% of the cad6-KD cysts formed tubules but <1% had filled lumens, whereas 100% of the Ecad-KD cysts had filled lumens, but no tubules were observed (Figure 1J).

Cell polarization on loss of cadherins

Confocal immunofluorescence microscopy confirmed that cadherin-6 and/or E-cadherin were effectively down-regulated in the cad6-KD, Ecad-KD, and 6/E-KD cysts (Figure 2, A–D). We next analyzed how cadherin knockdown affected cell polarization.

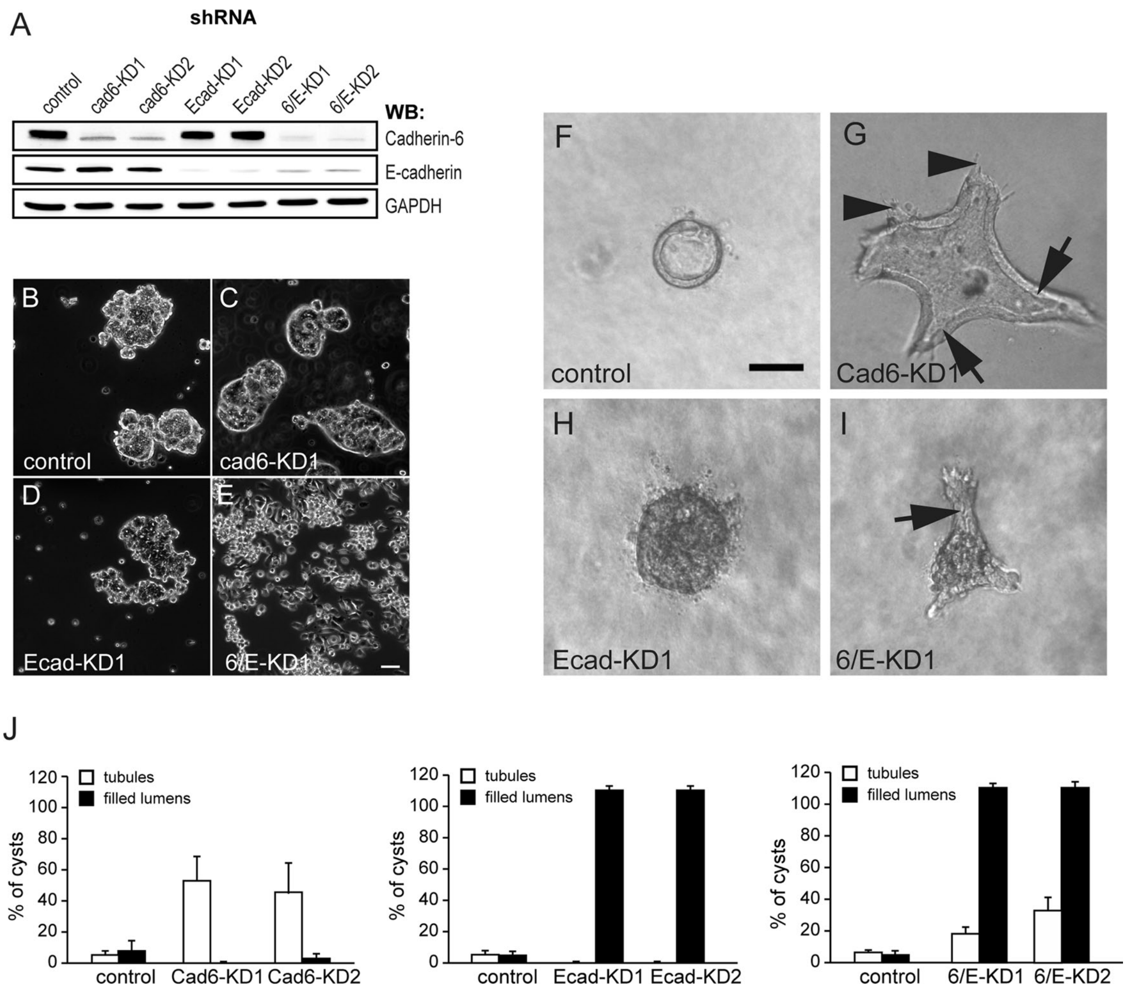

FIGURE 1: Knockdown of E-cadherin or cadherin-6 induces distinct phenotypes. MDCK cells expressing a nontargeting control vector or inducible shRNA against E-cadherin, cadherin-6, or both were grown in the presence of doxycycline to induce shRNA expression. (A) Western blots from lysates of confluent cells that were induced for 2 d to express a nontargeting shRNA (control) or different shRNAs against cadherin-6 (cad6-KD1, cad6-KD2), E-cadherin (Ecad-KD1, Ecad-KD2), or both (6/E-KD1, 6/E-KD2). (B–E) Cell–cell adhesion as analyzed by an aggregation assay in control (B), cad6-KD1 (C), Ecad-KD1 (D), or 6/E-KD1 (E) cells. (F–I) Control cells or cadherin knockdown cells were grown as cysts in collagen gels. Phase contrast images were taken 13 d after plating. Control cells (F) form cysts with a spherical phenotype and a hollow lumen. Cad6-KD cysts (G) develop large tubules (arrows) and some narrow extensions (arrowhead), whereas Ecad-KD cysts (H) form spherical cysts with filled lumens. 6/E-KD cysts (I) have both tubules and filled lumens (arrow). Scale bar, 50 µm. (J) Percentage of cysts with tubules and filled lumens in cad6-KD1, cad6-KD2 (left), Ecad-KD1, Ecad-KD2 (middle), and 6/E-KD1, 6/E-KD2 cysts (right). Data represent mean ± SD. N = 3.

Knockdown of cadherin-6 did not alter the basolateral localization of E-cadherin (compare Figure 2, A2 to B2) and vice versa (compare Figure 2, A1 to C1), and in both cases, β-catenin was still localized to the basolateral membrane (Figure 3, B1, C1). This indicates that cadherin-6 and E-cadherin play redundant roles in recruiting β-catenin to the basolateral membrane. The absence of basolateral β-catenin in 6/E-KD cysts (Figure 3D1) suggests that knockdown of both E-cadherin and cadherin-6 is sufficient to disrupt adherens junctions and that this defect is not compensated for by other cadherins. Cells in MDCK cysts orient their apical surface toward the central lumen. Apical polarization was not altered in cad6-KD cells, as judged by staining for the apical marker protein gp135/podocalyxin and the tight junction marker ZO-1 (compare Figure 3, A3, A4 to B3, B4). Ecad-KD cysts had multiple small lumens (Figure 3C3), which were not discernible by phase-contrast imaging (Figure 1D). Only cells facing these lumens exhibited apical-basolateral polarization of β-catenin, ZO-1, and gp135 (Figure 3, C1–C4). Apical-basolateral polarization was completely disrupted in 6/E-KD cysts, as judged by disorganized or loss of staining of β-catenin, ZO-1, and gp135 (Figure 3, D1–D4).

Tubulogenesis induced by HGF and DN-Pak1 coincides with down-regulation of cadherin 6

As knockdown of caderin-6 promoted the spontaneous formation of tubules, we investigated whether tubulogenesis induced by extracellular signals depends on a loss of cadherin-6. Stimulation of MDCK cysts with HGF induces the formation of branching tubules that recapitulate aspects of kidney tubulogenesis in vivo (Montesano *et al.*, 1991b; Zegers *et al.*, 2003b). Tubulogenesis occurs in distinct stages (Pollack *et al.*, 1998). Initially, some cells form a basal extension. Next, chains of single cells or cords of two to three cells in diameter extend from the cyst body into the collagen. Finally, small

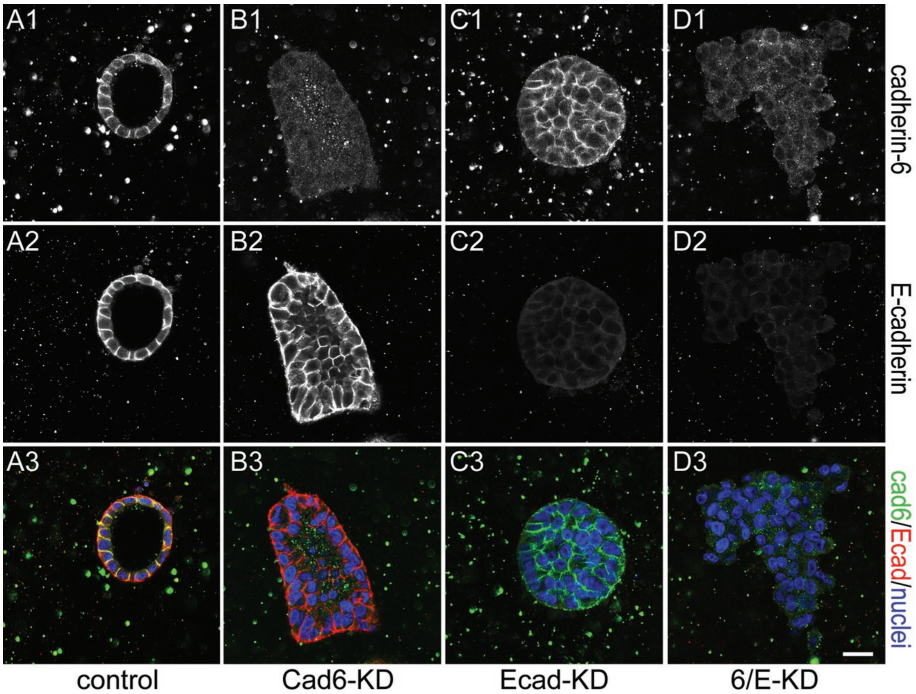

FIGURE 2: Specific knockdown of cadherin-6 or E-cadherin in 3D culture. (A1–D3) Control cysts (A1–A3) or cysts of MDCK cells expressing shRNA against cadherin-6 (B1–B3), E-cadherin (C1–C3), or both (D1–D3) were grown in the presence of doxycycline in 3D. Cysts were fixed and stained for E-cadherin and cadherin-6 at 13 d after plating. In control cysts, both cadherin-6 (A1) and E-cadherin (A2) localize at the lateral membrane. In cad6-KD cysts, cadherin-6 is lost from the lateral membrane (B1) without affecting expression or localization of E-cadherin (B2). Ecad-KD cysts have largely reduced E-cadherin (C2) at the basolateral surface, but expression or localization of cadherin-6 is not affected (C1). 6/E-KD cysts have lost expression of both cadherin-6 (D1) and E-cadherin (D2). Merged images (A3, B3, C3, D3) show cadherin-6 in green and E-cadherin in red. Nuclei, stained with 4′,6-diamidino-2-phenylindole (DAPI), are blue. Scale bar, 20 μm.

nascent lumens form within cords, which merge and eventually become continuous with the central lumen. During this process, E-cadherin becomes randomly distributed at the plasma membrane but remains at the plasma membrane at all times (Pollack *et al.*, 1998). Accordingly, cell–cell contact is maintained throughout tubulogenesis. We used conditioned media from MRC5 fibroblasts as the source of HGF to induce tubulogenesis (Montesano *et al.*, 1991a; Yu *et al.*, 2003). In agreement with previous reports (Pollack *et al.*, 1998; Leroy and Mostov, 2007), E-cadherin was maintained at the membrane in the HGF-induced tubules 24 h after stimulation (compare Figure 4, B2 and B3 and C2 and C3, to A2 and A3). In contrast, cadherin-6 was lost from the plasma membrane, and this was often (Figure 4C1), but not always (Figure 4B1) restricted to cells within cords and chains.

When we compared the phenotypes of tubules induced by HGF and those in the cad6-KD cyst, we noticed morphological distinctions. In contrast to the HGF-induced tubules, the cad6-KD–induced tubules mainly exhibit either small extensions or large lumen-containing tubules but very few chains and cords (Figure 1C). We recently reported a remarkably similar phenotype in cells that inducibly express a kinase-dead, dominant-negative mutant of Pak1 (Pak1-K299R) (Hunter and Zegers, 2010). In this Tet-off system, Pak1-K299R is expressed under the control of the tetracycline-controlled transactivator. Expression of Pak1-K299R is suppressed by doxycycline and induced by removing doxycycline from the culture media. As we found previously, Pak1-K299R–expressing cysts (−dox) formed robust tubules, which maintained the lateral localization of E-cadherin (compare Figure 5, A2 to B2) (Hunter and Zegers, 2010). Cadherin-6, however, was completely lost from the membrane in all cells. This was likely due to down-regulation of cadherin-6 expression, as Western blot analysis showed a large decrease in the levels of cadherin-6 but not E-cadherin (Figure 5C). To analyze whether the loss of cadherin-6 was required for Pak1-K299R–induced tubules, we next restored cadherin-6 expression by stably expressing EGFP-tagged mouse cadherin-6 in these cells (Figure 6A). Examination of live cysts revealed that cadherin-6–EGFP expression did not alter cyst morphology in the absence of Pak1-K299R expression (+dox; compare Figure 6, B to D1) and that cadherin–EGFP exclusively localized to the lateral membranes (Figure 6D2). Cadherin-6–EGFP also localized laterally in Pak1-K299R–expressing cells (Figure 6C2) and strongly inhibited Pak1-K299R–induced tubules (−dox, compare Figure 6, C to E1). Quantification of the cyst phenotypes revealed that expression of cadherin-6–EGFP completely inhibited the increased tubulogenesis that was induced by Pak1-K299R expression (Figure 6F, compare +dox and −dox). Western blot analysis showed that at least one of the clones (K299R-cad6-EGFP#1) expressed equal levels of Pak1-K299R as compared with the parental cells, although expression levels of clone K299R-cad6-EGFP#2 were somewhat lower (Figure 6A). We previously found that Pak1-K299R clones with expression levels that were at least 50% lower than the clone shown in Figure 6, C and F, still induce robust tubules (M.Z., unpublished data), and even clones with Pak1-K299R expression that is below the detection limit in Western blots could induce tubules. The generally higher levels of tubulogenesis in the noninduced K299R-cad6-EGFP clones, although not statistically significant, may therefore be explained by “leakiness” of the Pak1-K299R expression in these clones. For the same reason, it is unlikely that the lower expression levels of Pak1-K299R in the K299R-cad6-EGFP#2 clone account for the inhibition of tubulogenesis. Rather, our data obtained with the two cadherin-6–overexpressing clones indicate that down-regulation of cadherin-6 is required for tubulogenesis in Pak1-K299R–expressing cysts.

PI3K is required for tubulogenesis in cad6-KD cysts

HGF activates the PI3K and ERK pathways, which are both required for the initial stages of HGF-induced tubulogenesis (Rosario and Birchmeier, 2003; Yu *et al.*, 2003; O’Brien *et al.*, 2004). We analyzed whether these pathways were activated in cad6-KD cysts, using phospho-specific antibodies against the activated forms of ERK (phospho-Thr202, Tyr204-ERK1,2, p-ERK) and Akt (phospho-Ser473-Akt, p-Akt). Increased levels of p-Akt and p-ERK suggested that both PI3K and ERK signaling was upregulated in cad6-KD cysts (Figure 7A). We next used pharmacological inhibitors to test whether PI3K and ERK are required for tubulogenesis. To do so, we inhibited PI3K and ERK in cad6-KD cysts with the selective inhibitors LY294002 and PD98059, respectively. We and others previously showed that

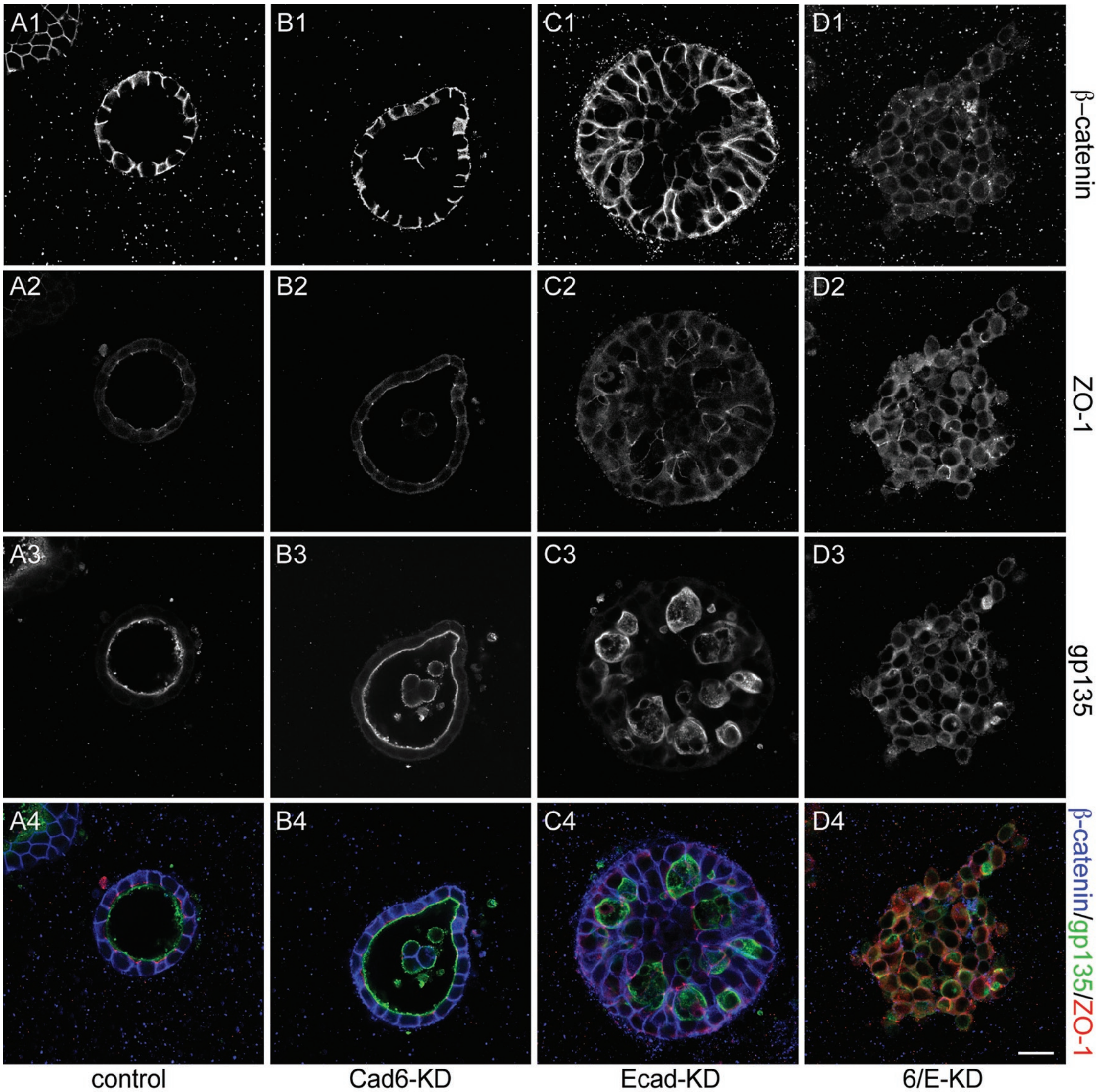

FIGURE 3: Knockdown of cadherin-6, E-cadherin, or both differentially affects cell polarization. (A1–D4) Control and cadherin knockdown cysts were plated and grown as described for Figure 2. Cysts were stained for β-catenin (A1, B1, C1, and D1), ZO-1 (A2, B2, C2, and D2) and gp135 (A3, B3, C3, and D3). Merged images of β-catenin (blue), gp135 (green), and ZO-1 (red) are also shown (A4, B4, C4, and D4). Scale bar, 20 µm.

cysts can be grown in the presence of 20 µM LY294002 (Yu *et al.*, 2003) or ERK inhibitors (O'Brien *et al.*, 2004; unpublished data) for at least 3 d without leading to appreciable cell death. When we treated 13-d-old cad6-KD cysts for 12 h with 20 µM LY294002 or PD98059, the increased PI3K and ERK activation, respectively, was greatly reduced (Figure 7B). Inhibition of PI3K with LY294002 for up to 2 d dramatically inhibited tubules in cad6-KD cysts (Figure 7C). Identical results were obtained with an alternative PI3K inhibitor, ZSTK474 (Yaguchi *et al.*, 2006), at a concentration of 500 nM (unpublished data). In contrast, even though the percentage of tubule-forming cyst in PD98059-treated cultures was lower in some individual experiments, the decrease was highly variable and not statistically significant (Figure 7C). Of interest, none of the ERK and PI3K inhibitors rescued the filled lumens in the Ecad-KD cysts (Figure 7D), again emphasizing the distinct functions of E-cadherin and cadherin-6.

To better understand the requirement of PI3K for tubulogenesis in cad6-KD cysts, we examined the dynamics of tubules in individual cysts over the time course of LY294002 or vehicle treatment (control). For this, we used a gridded cover slip mounted under the bottom of the cyst culture for orientation purposes. Cysts were imaged daily starting at day 13, at which time LY294002 or vehicle was added, and ended 48 h later, at day 15. In general, most of the tubules formed in vehicle cad6-KD cysts over this time course were either stable or elongated or changed shape over the 48-h time course (Figure 7E, top two rows). In contrast, tubule formation or elongation in LY294002-treated cysts was initially inhibited, which was followed by a shortening and contraction of tubules. After 48 h, most cysts had reverted to spherical shape (Figure 7E, bottom two rows), consistent with our quantification data (Figure 7C). Taken together, these data suggest that PI3K is required for the formation and maintenance of tubules that form on ablation of cadherin-6.

DISCUSSION

Cadherins are crucial for tissue morphogenesis in many different contexts (Gumbiner, 2005). Studies on the function of classic cadherins in epithelial homeostasis and polarization have almost exclusively focused on the predominant family member, E-cadherin. It is

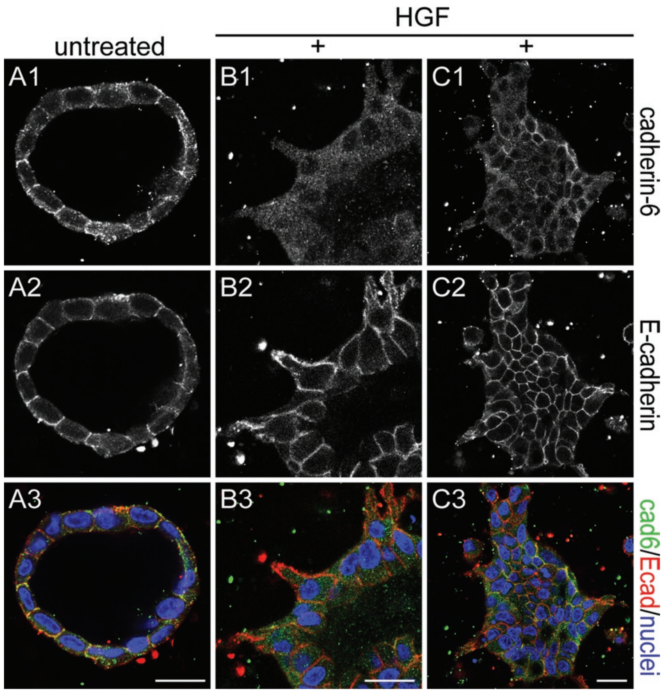

FIGURE 4: Cadherin-6 is lost from the membrane in early HGF-induced tubules. (A1–C3) Control cysts were grown for 13 d and were left untreated (control, A1–A3) or were treated for 24 h with HGF-containing conditioned medium (HGF, B1–C3). Cysts were fixed and stained for cadherin-6 (A1, B1, C1) and E-cadherin (A2, B2, C2). Note a decrease of cadherin-6 from the membrane either in larger areas of HGF-treated cysts (B1) or specifically in cords or chains (C1). In all cases, localization of E-cadherin (B2, C2) is not changed as compared with control (A2). Merged images (A3, B3, C3) show cadherin-6 in green and E-cadherin in red. Nuclei, stained with DAPI, are in blue. Scale bar, 20 µm.

surprising, however, that ablation of E-cadherin in MDCK cells, a popular model to study the roles of E-cadherin in cell polarization, does not induce gross changes in 2D culture (Capaldo and Macara, 2007; den Elzen *et al.*, 2009; Liu *et al.*, 2010). This has led to the suggestion that cadherin-6 compensates for a loss of E-cadherin in these cells and that both cadherins have largely redundant roles (Capaldo and Macara, 2007; den Elzen *et al.*, 2009). Here we used a 3D cell culture model to analyze the roles of both cadherins. We demonstrate that in 3D cell culture cadherin-6 and E-cadherin play specific, nonredundant roles. Specifically, we show that cadherin-6 functions as an inhibitor of tubulogenesis but is not required for cell polarization. In contrast, E-cadherin is required to form single lumens.

Cadherin-6 inhibits tubulogenesis but is dispensable for cell polarization or lumen formation. Conditions that induce tubulogenesis, such as treatment with HGF (Yu *et al.*, 2003) or expression of Pak1-K299R (Hunter and Zegers, 2010), down-regulated basolateral cadherin-6 but not E-cadherin. This change was the largest in cysts expressing Pak1-K299R, which form large tubules (Hunter and Zegers, 2010) that resemble those in cad6-KD cysts and could be blocked by overexpressing EGFP-tagged cadherin-6. Depletion of cadherin-6 activated both the ERK and PI3K signaling pathways, which are also required and sufficient for HGF-induced tubulogenesis (Cantley *et al.*, 1994; Khwaja *et al.*, 1998; Yu *et al.*, 2003; O'Brien *et al.*, 2004). We show here that PI3K but not ERK activity was required for tubulogenesis as induced by cadherin-6 depletion. Together, our data are consistent with a model in which cadherin-6 inhibits tubulogenesis by repressing PI3K activation. Of interest, we

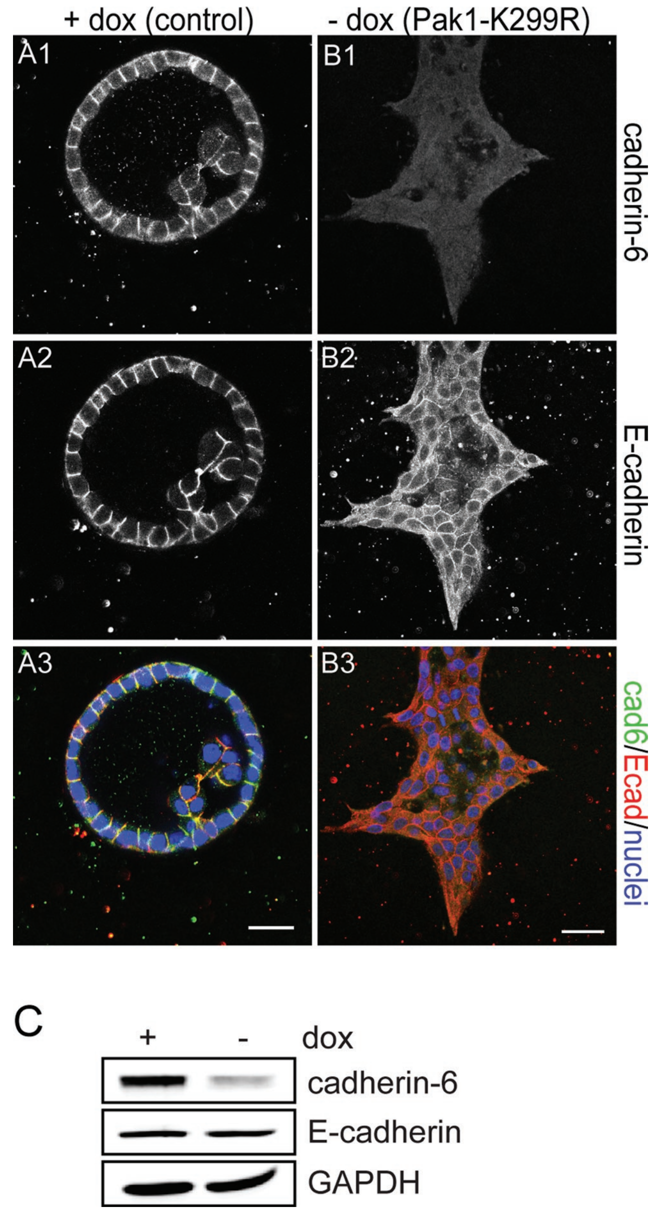

FIGURE 5: Expression of Pak1-K299R down-regulates cadherin-6 and induces tubulogenesis. (A1–A3) In the presence of doxycycline (+dox), control cells form cysts with a normal spherical morphology, a central lumen, and cadherin-6 (A1, green in A3) and E-cadherin (A2, red in A3) at the basolateral membrane. (B1–B3) In the absence of doxycycline (−dox), Pak1-K299R is expressed and cysts form tubules and have lost expression of cadherin-6 (B1, green in B3) but not E-cadherin (B2, red in B3). Nuclei, stained with DAPI, are blue (A3, B3). Scale bar, 20 µm. (C) Western blot of lysates of control (+dox) and Pak1-K299R–expressing cells were stained for cadherin-6 and E-cadherin. GAPDH is a loading control.

recently showed that Pak1-K299R also activated PI3K, by a mechanism that involved cadherin-mediated cross-talk between cell–cell and cell–matrix adhesions (Liu *et al.*, 2010). As these studies were done with a dominant-negative E-cadherin, either cadherin-6 or E-cadherin could be involved in this regulation, and it would be tempting to speculate that cadherin-6 may be specifically involved in this cross-talk.

Cadherin-6 is highly expressed during embryonic kidney development and is required for nephrogenesis in zebrafish (Kubota

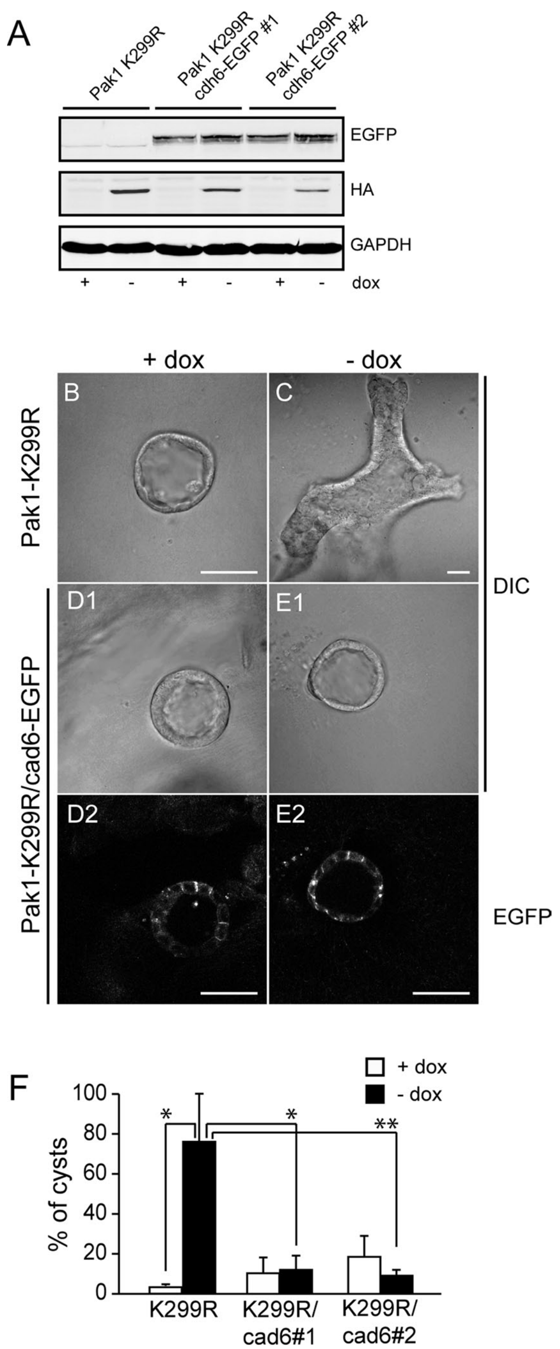

FIGURE 6: Tubulogenesis in Pak1-K299R–expressing cells is inhibited by overexpression of EGFP-tagged cadherin-6. (A) Western blot of showing expression levels of EGFP-tagged cadherin-6 in parental Pak1-K299R–expressing cells and two clones constitutively expressing cadherin-6–EGFP. Note that the expression of HA-tagged Pak1-K299R is effectively suppressed by the presence of doxycycline and induced in the absence of doxycycline. (B, C) Differential interference contrast images of live cells show that Pak1-K299R cells grown in the presence of doxycycline have normal spherical phenotype (B) and form tubules in the absence of doxycycline (C). (D1–E2) Cadherin-6–EGFP localizes to the lateral surface of Pak1-K299R cells in both the presence (D2) and absence (E2) of doxycycline and inhibits Pak1-K299R–induced tubulogenesis. (F) Quantification of tubulating cysts in Pak-K299R cells and clones expressing cadherin-6–EGFP. Data represent mean ± SD. N = 3. *p < 0.02, **p < 0.01. Other differences between cells grown with or without doxycycline, or between noninduced cells, were not significant.

et al., 2007) and mice (Mah *et al.*, 2000). It promotes MET during nephrogenesis (Cho *et al.*, 1998; Dahl *et al.*, 2002), as *Cdh6*-null kidneys are delayed in MET (Mah *et al.*, 2000), whereas a cad6-inhibitory antibody inhibits MET in embryonic organ culture (Cho *et al.*, 1998). In contrast, inhibition of E-cadherin does not inhibit kidney tubulogenesis in vitro (Falk *et al.*, 1996). In MDCK cells, a partial EMT mediates HGF-induced tubulogenesis, followed by a repolarization stage that resembles MET (O'Brien *et al.*, 2004). It is therefore possible that cadherin-6 inhibits tubulogenesis by promoting MET and inhibiting EMT, although it is important to point out that nephrogenesis in vivo involves MET in the metanephric mesenchyme but not the tubular epithelia (Nigam and Shah, 2009).

Consistent with this, we find that ablation of E-cadherin does not induce tubulogenesis. Instead, Ecad-KD cysts had multiple small lumens, and only the small fraction of cells surrounding these lumens polarized and formed an apical surface. Similar multilumenal phenotypes have been associated with defects in apicobasolateral polarization and/or the orientation of mitotic spindles in MDCK and Caco-2 cells (Bryant and Mostov, 2008; Jaffe *et al.*, 2008; Schluter *et al.*, 2009; Qin *et al.*, 2010; Rodriguez-Fraticelli *et al.*, 2010). In normal cysts, the mitotic spindle aligns parallel to the cyst monolayer, which promotes cyst expansion while maintaining a single lumen. Inhibition of cdc42 (Jaffe *et al.*, 2008; Qin *et al.*, 2010; Rodriguez-Fraticelli *et al.*, 2010) or the polarity protein Par3 (Hao *et al.*, 2010) randomizes this orientation, causing the formation of multiple small lumens. DN-E-cadherin also induces partially filled lumens in 3D (Troxell *et al.*, 2001) and defects in spindle orientation in 2D, and this latter defect could be rescued by E-cadherin (den Elzen *et al.*, 2009). Defects in mitotic spindle orientation may therefore cause the multilumenal phenotype upon E-cadherin knockdown, although polarity defects that occur independent of the spindle cannot be excluded.

We show that E-cadherin and cadherin-6 serve unique functions and that their expression levels are regulated independently, particularly during tubulogenesis. An earlier study showed that cadherin-6 and E-cadherin are part of distinct, mutually exclusive β-catenin–containing protein complexes that form with different kinetics (Stewart *et al.*, 2000). This study also showed that at least partially distinct mechanisms control the expression levels of both cadherins and that cadherin-6 levels are regulated strictly at the posttranslational level. Although the mechanism of cadherin-6 destabilization was not elucidated, truncated β-catenin mutants that stimulate canonical Wnt signaling enhanced the degradation of cadherin-6 (Stewart *et al.*, 2000). It must be noted, however, that those mutants inhibit rather than stimulate HGF-induced tubulogenesis (Pollack *et al.*, 1997), which suggests that under these conditions destabilization of cadherin-6 is not sufficient to promote tubulogenesis. The structural differences between cadherin-6 and E-cadherin that control its differences in stability and/or localization are still unclear. Furthermore, our work has not elucidated whether the ability of cadherin-6 to inhibit tubulogenesis requires its adhesive capabilities or relies solely on its cytoplasmic domain. Both cadherins have conserved β-catenin– and p120-catenin–binding domains (Nollet *et al.*, 2000), and our preliminary experiments indicate they interact with β-catenin or p120-catenin, respectively, to a similar extent (F.L. and M.Z., unpublished data). Furthermore, we show that both cadherins recruit β-catenin or p120-catenin to the basolateral surface and can mediate cell–cell adhesion, at least as judged by an aggregation assay. E-cadherin can be down-regulated by interacting with the E3 ubiquitine ligase Hakai. Hakai binds a tyrosine-based motif in the E-cadherin C-terminus after phosphorylation of this motif by Src (Fujita *et al.*, 2002). Cadherin-6 lacks this motif,

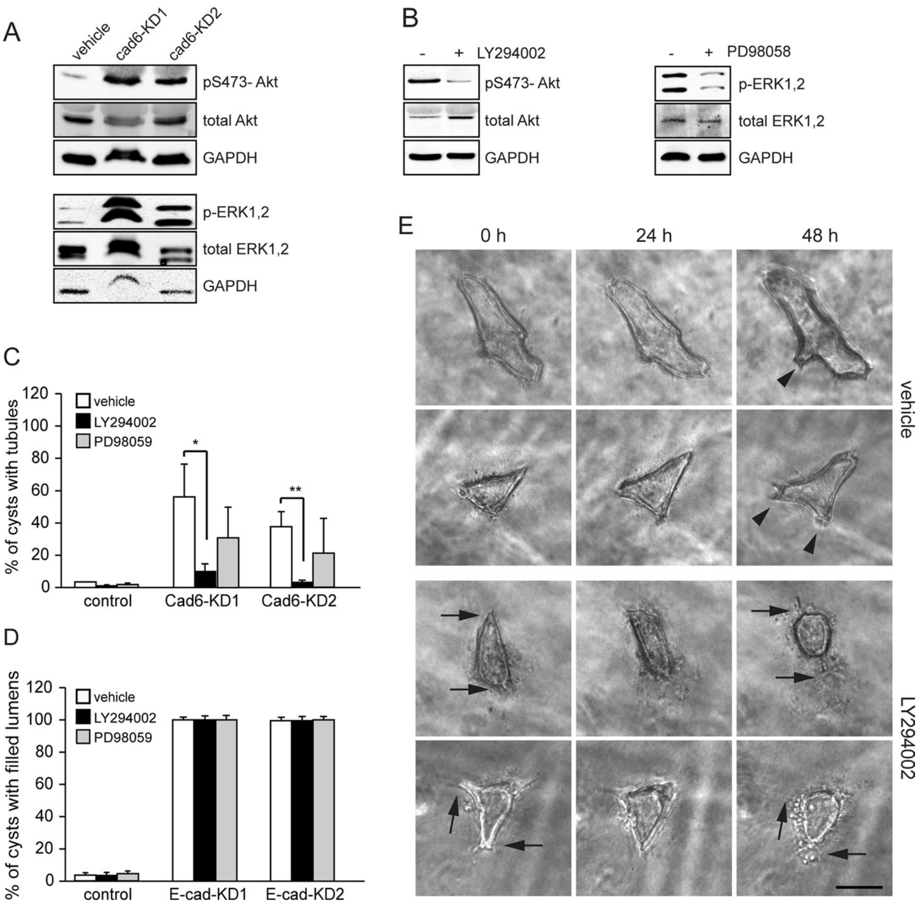

FIGURE 7: Tubulogenesis in cad6-KD cysts depends on PI3K but not ERK activation. (A) Western blots from lysates of control and cad6-KD cysts show that knockdown of cadherin-6 increases activation of both the PI3K and ERK pathways as judged by increased staining of Akt at Ser473 (pS473-Akt) and phosphorylation of p44/p42 ERK1/2 at Thr202/Tyr204. (B) LY294002 and PD98058 effectively inhibit PI3K and ERK activation in cad6-KD cysts when treated at concentrations of 20 μM for 12 h. (C, D) Quantification of tubulogenesis cad6-KD cysts (C) and lumen filling in Ecad-KD cysts (D) after a 48 h or treatment with vehicle (control, white bars), 20 μM LY294002 (black bars), or 20 μM PD98058 (gray bars). Cysts were treated 13 d after plating. Data show percentage of cad6-KD cysts with tubules (C) or Ecad-KD cysts with filled lumens (D) and represent mean data ± SD. N = 4. *$p < 0.01$, **$p < 0.05$. (E) Cad6-KD1 cysts treated with vehicle or 20 μM LY294002 for 48 h as described in the legend of Figure 6B. Phase-contrast images of individual cysts were taken before and 24 and 48 h after treatment. Two representative time series for control and cad6-KD1 cysts are shown. Arrowheads in control indicate tubules that elongate over time. Arrows mark tubules that contract 24 h after treatment with LY294002 and disappear at 48 h, leading to the restoration of a spherical cyst phenotype. Scale bar, 50 μm.

and it is therefore unlikely that Hakai is involved in destabilizing cadherin-6 downstream of HGF or in Pak1-K299R–expressing cells. It is also possible that the preferential destabilization of cadherin-6 does not exclusively rely on differences in the primary structure of E-cadherin and cadherin-6. Indeed, overexpression of DN-E-cadherin, which comprises the E-cadherin cytoplasmic domain, downregulates both E-cadherin and cadherin-6 (Liu *et al.*, 2010). This indicates that a protein that binds and is sequestered by the E-cadherin cytoplasmic domain can stabilize cadherin-6.

Our findings that E-cadherin and cadherin-6 serve unique functions in MDCK cells are relevant in a broader context. Most epithelia will not express cadherin-6, as its expression is mainly restricted to the kidney and areas of the central nervous system (UniGene, National Center for Biotechnology Information, Bethesda MD). Thus many epithelial cells may solely rely on E-cadherin to regulate different aspects of epithelial morphogenesis. Several of the cellular processes that are regulated by E-cadherin are strongly interdependent, particularly apicobasolateral polarization and cell–cell adhesion. This has hampered studies that aim to analyze the role of E-cadherin in these processes in isolation. Hence understanding the molecular mechanisms that drive the unique phenotypes that are induced upon the loss of either E-cadherin or cadherin-6 in MDCK cells will allow us to delineate general intracellular pathways by which epithelial cadherins regulate epithelial homeostasis.

MATERIALS AND METHODS

Antibodies and reagents

LY294002, ZSTK474, and PD98059 were from LC Laboratories (Woburn, MA). Polyclonal rabbit cadherin-6 antibodies specific against cadherin-6 were generated by Open Biosystems (Huntsville, AL) and raised against the peptide PHVEPRFLYLGPFKDSC (Stewart *et al.*, 2000) coupled to keyhole limpet hemocyanin. Cadherin-6 antiserum was affinity purified with the immunogenic peptide using the SulfoLink Immobilization kit for peptides (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Goat anti–cadherin-6 and rabbit β-catenin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Rat and mouse monoclonal E-cadherin antibody were from Sigma-Aldrich (St. Louis, MO) and BD, respectively. Mouse anti-gp135 was a kind gift from George Ojakian (SUNY, Downstate Medical Center, Brooklyn, NY). The mouse monoclonal anti–glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Biodesign International (Saco, ME). Rat anti-ZO1 was obtained via the Developmental Studies Hybridoma Bank at the University of Iowa. Rabbit anti-Akt, p-S473-Akt, ERK, phospho-Thr202, and Tyr204-ERK1, 2 antisera were from Cell Signaling Technology (Beverly, MA). Secondary antibodies for fluorescence microcopy comprised Alexa Fluor 488 donkey anti–mouse immunoglobulin G (IgG) (H+L), anti-rat, and anti-rabbit conjugates, Alexa Fluor 555 donkey anti–mouse IgG (H+L) and anti-rabbit conjugates, and Alexa Fluor 633 goat anti–rat IgG (H+L) (Invitrogen, Carlsbad, CA). LI-COR 800 secondary antibodies were from LI-COR Biosciences (Lincoln, NE), and horseradish peroxidase–conjugated antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Expression constructs and vector-based shRNA gene suppression cloning

An expression construct for mouse cadherin-6 (*Cdh6*) was provided by Takayoshi Inoue (National Institute of Neuroscience, Tokyo, Japan). To generate EGFP-tagged cadherin-6, cadherin-6 was excised from pCA-mcad6-HA-pA with *Hin*dIII/*Xma*I and ligated into pEGFP-N2 that was also cut with *Hin*dIII/*Xma*I. The cadherin-6-EGFP was cut out from this construct using *Hin*dIII/*Xba*I and ligated into pCB7 also cut with *Hin*dIII/*Xba*I. Conditional shRNA-based knockdown of cadherin expression in MDCK cells was performed as described previously (Tzaban *et al.*, 2009). Briefly, complementary 80–base pair oligonucleotides containing *Bam*HI and *Hin*dIII overhangs (Sigma-Aldrich) were annealed and inserted into pReSI-H1-puro. To generate cell lines in which both cadherins could be knocked down, oligonucleotides targeting E-cadherin were also inserted into pReSI-H1-hygro. Target shRNA sequences were Cad6-KD1:GAACTTACCGGTACTTCTTGC; Cad6-KD2:GCAGGAGATCTCTTCATTATC; Ecad-KD1:GTGACAGATCAGAATGACAAC; and Ecad-KD2: GGAGGTTAATCCAGAATCTGG.

Cell culture and retroviral packaging

MDCK cells that conditionally express Pak1-K299R using the Tet-off system were described previously (Zegers *et al.*, 2003a). Clones of Pak1-K299R cells expressing EGFP-tagged cadherin-6 were generated by transfecting these cells with EGFP-cadherin-6 in pCB7 using calcium phosphate transfection and selection with hygromycin B. MDCK TR-T10 cells, which express the Tet repressor, were kindly provided by Sanjay Pimplikar (Gao and Pimplikar, 2001). MDCK cells were grown in MEM, supplemented with 10% fetal calf serum (FCS) and penicillin–streptomycin. The Phoenix packaging cell line was grown in DMEM with 10% FCS and penicillin–streptomycin.

To generate cadherin knockdown cells, shRNA-containing vectors were transfected into the Phoenix packaging cells using FuGene6, and virus-containing medium was used to transduce cells. For transduction, MDCK TR-T10 cells were seeded onto six-well tissue culture plate and media containing recombinant retroviruses, and Polybrene at a final concentration of 5 µg/ml was added to the cells 16 h later. After 24 h of incubation, the medium was replaced with normal growth media. After 2 d, the cells were split and selected with 2.5 µg/ml of puromycin for 14 d. Single colonies were further analyzed by Western blot and immunofluorescence for knockdown efficiency. Tet-inducible rescue cell lines for cadherin-6 knockdown were generated by introducing mutations in the cadherin 6–EGFP construct to make it refractory to cad6-KD1. The refractory cadherin-6–EGFP was subcloned into a retroviral vector containing the CMV promoter under control of the $TetO_2$ operator with hygromycin B as a selection marker (pQCTXH, vector developed by S. H. Hansen). Clones were selected with 150 µg/ml of hygromycin B. To generate cells in which both E-cadherin and cadherin-6 could be knocked down (6/E-KD), clones with efficient cadherin-6 knockdown (Cad6-KD1) were transduced with retroviruses expressing Ecad-KD2 in pReSI-H1-hygro. Clones were selected with 150 µg/ml of hygromycin B. Expression of the shRNA hairpin was induced by addition of 4 µg/ml of doxycycline to the growth media. All cells were maintained in a humidified atmosphere at 37°C in 5% CO_2 and 95% air.

3D culture of cysts

3D culture of MDCK cells in collagen was performed as described previously (O'Brien *et al.*, 2006). Briefly, single cells were suspended at a concentration of 2×10^4/ml in a collagen I solution containing 2 mg/ml of collagen I (PureCol; Advanced Biomatrix, San Diego, CA), 0.23% $NaHCO_3$ wt/vol, and 20 mM HEPES (pH 7.6) in growth medium with or without doxycycline. For inhibitors and HGF stimulation experiments, 150 µl of cell–collagen mixture was plated onto Nunc Anopore membrane inserts with 0.02-µm pore size and 10-mm diameter (Nalge Nunc International, Rochester, NY) in a 24-well tissue culture plate. To analyze phenotypes and to prepare cell lysates, gels were plated in 96-well tissue culture plates. To analyze protein levels, the cell concentration was increased to 5×10^4 cells/ml and an additional cell-free collagen gel was plated at the bottom of the well to avoid a contribution of cells grown as a monolayer underneath the gel. Knockdown of the target protein was induced at the same time as plating in collagen by the addition of doxycycline at final concentration of 4 µg/ml. To quantify cyst phenotypes, cysts were examined by phase-contrast microscopy, and 75–100 cysts were scored for each condition. To monitor individual cysts over time, cover slips with numbered grid patterns (Electron Microscopy Sciences, Hatfield, PA) were glued to the bottom of a 96-well tissue culture plate as a reference, and 5–10 cysts were photographed at the indicated time points.

Aggregation assay

Aggregation assays were done essentially as described previously (Qin *et al.*, 2005). Briefly, single cell suspensions were prepared by incubating cells with 4 mM EDTA and 1 mM EGTA in phosphate-buffered saline (PBS). Next, cells were resuspended in growth medium and 6×10^4 cells in a 30-µl drop were allowed to aggregate for 18 h at 37°C in a drop hanging from the lid of a multidish plate. Cells were then subjected to shear force by pipetting 10 times using a 200-µl pipette tip. Cell aggregates were images by phase contrast, using a 10× objective.

Western blotting of cysts samples

Collagen gels with cysts were collected and lysed with 40 µl of 4× Laemmli buffer, mixed, boiled for 5 min, and sonicated for 20 s. Samples were normalized by gel electrophoresis and quantitative immunoblotting with GAPDH antibodies using an Odyssey detector (LI-COR). Next, samples containing equal amounts of cellular protein were loaded onto 10% SDS–polyacrylamide gels, separated, and transferred onto polyvinylidene fluoride membrane (Millipore, Billerica, MA). To confirm equal loading, GAPDH was again used as a loading control.

Immunofluorescence microscopy

The protocol for immunofluorescence staining of cysts was previously described in detail (O'Brien *et al.*, 2006). Briefly, collagen gels were rinsed twice in PBS plus Ca^{2+} and Mg^{2+} (PBS+) and first briefly digested with 50 U/ml of collagenase (type IA; Sigma) in PBS+ at 37°C for 10 min in the presence of protease inhibitors to inhibit contaminating proteases. Cells were then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and quenched in 50 mM NH_4Cl in PBS for 30 min. Cysts samples were blocked in 5% normal donkey serum in PBS/TX-100 for another 30 min at room temperature. Primary antibodies were added to cyst samples to incubate overnight at 4°C with rotating. After extensive washing with PBS/TX-100, the samples were incubated with secondary antibody Fluorsave (Calbiochem, La Jolla, CA) at a 1:250 dilution overnight at 4°C with rotating. Finally, the gels were washed in PBS/TX-100 four times, followed by PBS once and dH_2O once, and then mounted on glass slides with 10 µg/ml of 4′-6-diamidino-2-phenylindole (DAPI) to stain nuclei. Cysts were photographed on a Zeiss 510 LSM confocal microscope with an Axiovert 200M microscope and a C-Apochromat x63/1.2W Corr lens. Images were adjusted for brightness with Photoshop CS, version 11.0 (Adobe, San Jose, CA).

Statistical analyses

Data shown as mean ± SD are from at least three experiments. Differences between two groups were examined in Excel (Microsoft, Redmond, WA) using an unpaired *t* test. We applied a significance level of $p < 0.05$ for all statistical analyses.

ACKNOWLEDGMENTS

We thank Cassandra Kontur and Fernando Vazquez for excellent technical assistance. Takayoshi Inoue is gratefully acknowledged for providing the mouse cadherin-6 expression construct. This work was supported by National Institutes of Health Grants R01 CA142647 (to S.H.H.) and R01 GM076363 (to M.Z.).

REFERENCES

Adams CL, Nelson WJ, Smith SJ (1996). Quantitative analysis of cadherin–catenin–actin reorganization during development of cell–cell adhesion. J Cell Biol 135, 1899–1911.

Affolter M, Zeller R, Caussinus E (2009). Tissue remodelling through branching morphogenesis. Nat Rev Mol Cell Biol 10, 831–842.

Benjamin JT, Gaston DC, Halloran BA, Schnapp LM, Zent R, Prince LS (2009). The role of integrin alpha8beta1 in fetal lung morphogenesis and injury. Dev Biol 335, 407–417.

Bracke ME, Van Roy FM, Mareel MM (1996). The E-cadherin/catenin complex in invasion and metastasis. Curr Top Microbiol Immunol 213 (Pt 1), 123–161.

Bryant DM, Mostov KE (2008). From cells to organs: building polarized tissue. Nat Rev Mol Cell Bio 9, 887–901.

Cantley LG, Barros EJ, Gandhi M, Rauchman M, Nigam SK (1994). Regulation of mitogenesis, motogenesis, and tubulogenesis by hepatocyte growth factor in renal collecting duct cells. Am J Physiol Cell Physiol 267, F271–F280.

Capaldo CT, Macara IG (2007). Depletion of E-cadherin disrupts establishment but not maintenance of cell junctions in Madin-Darby canine kidney epithelial cells. Mol Biol Cell 18, 189–200.

Cho EA, Patterson LT, Brookhiser WT, Mah S, Kintner C, Dressler GR (1998). Differential expression and function of cadherin-6 during renal epithelium development. Development 125, 803–812.

Dahl U, Sjodin A, Larue L, Radice GL, Cajander S, Takeichi M, Kemler R, Semb H (2002). Genetic dissection of cadherin function during nephrogenesis. Mol Cell Biol 22, 1474–1487.

den Elzen N, Buttery CV, Maddugoda MP, Ren G, Yap AS (2009). Cadherin adhesion receptors orient the mitotic spindle during symmetric cell division in mammalian epithelia. Mol Biol Cell 20, 3740–3750.

Ewald AJ, Brenot A, Duong M, Chan BS, Werb Z (2008). Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. Dev Cell 14, 570–581.

Falk M, Salmivirta K, Durbeej M, Larsson E, Ekblom M, Vestweber D, Ekblom P (1996). Integrin alpha 6B beta 1 is involved in kidney tubulogenesis in vitro. J Cell Sci 109, 2801–2810.

Fujita Y, Krause G, Scheffner M, Zechner D, Leddy HE, Behrens J, Sommer T, Birchmeier W (2002). Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. Nat Cell Biol 4, 222–231.

Gao Y, Pimplikar SW (2001). The gamma-secretase–cleaved C-terminal fragment of amyloid precursor protein mediates signaling to the nucleus. Proc Natl Acad Sci USA 98, 14979–14984.

Gumbiner BM (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 84, 345–357.

Gumbiner BM (2005). Regulation of cadherin-mediated adhesion in morphogenesis. Nat Rev Mol Cell Biol 6, 622–634.

Hao Y, Du Q, Chen X, Zheng Z, Balsbaugh JL, Maitra S, Shabanowitz J, Hunt DF, Macara IG (2010). Par3 controls epithelial spindle orientation by aPKC-mediated phosphorylation of apical pins. Curr Biol 20, 1809–1818.

Harris TJ, Tepass U (2010). Adherens junctions: from molecules to morphogenesis. Nat Rev Mol Cell Biol 11, 502–514.

Hunter MP, Zegers MM (2010). Pak1 regulates branching morphogenesis in 3D MDCK cell culture by a PIX and beta1-integrin–dependent mechanism. Am J Physiol Cell Physiol 299, C21–32.

Jaffe AB, Kaji N, Durgan J, Hall A (2008). Cdc42 controls spindle orientation to position the apical surface during epithelial morphogenesis. J Cell Biol 183, 625–633.

Jou TS, Stewart DB, Stappert J, Nelson WJ, Marrs JA (1995). Genetic and biochemical dissection of protein linkages in the cadherin–catenin complex. Proc Natl Acad Sci USA 92, 5067–5071.

Khwaja A, Lehmann K, Marte BM, Downward J (1998). Phosphoinositide 3-kinase induces scattering and tubulogenesis in epithelial cells through a novel pathway. J Biol Chem 273, 18793–18801.

Klinowska TC, Soriano JV, Edwards GM, Oliver JM, Valentijn AJ, Montesano R, Streuli CH (1999). Laminin and beta1 integrins are crucial for normal mammary gland development in the mouse. Dev Biol 215, 13–32.

Koyama N, Hayashi T, Gresik EW, Kashimata M (2009). Role of alpha 6 integrin subunit in branching morphogenesis of fetal mouse submandibular gland: investigation by mesenchyme-free epithelial culture system. J Med Invest 56(Suppl), 247–249.

Kubota F, Murakami T, Mogi K, Yorifuji H (2007). Cadherin-6 is required for zebrafish nephrogenesis during early development. Int J Dev Biol 51, 123–129.

Larue L, Ohsugi M, Hirchenhain J, Kemler R (1994). E-cadherin null mutant embryos fail to form a trophectoderm epithelium. Proc Natl Acad Sci USA 91, 8263–8267.

Leroy P, Mostov KE (2007). Slug is required for cell survival during partial epithelial–mesenchymal transition of HGF-induced tubulogenesis. Mol Biol Cell 18, 1943–1952.

Liu F, Jia L, Thompson-Baine A, Puglise JM, ter Beest MB, Zegers MM (2010). Cadherins and Pak1 control contact inhibition of proliferation by Pak1-βPIX–complex dependent regulation of cell-matrix signaling. Mol Cell Biol 30, 1771–1783.

Mah SP, Saueressig H, Goulding M, Kintner C, Dressler GR (2000). Kidney development in cadherin-6 mutants: delayed mesenchyme-to-epithelial conversion and loss of nephrons. Dev Biol 223, 38–53.

McCrea PD, Turck CW, Gumbiner B (1991). A homolog of the armadillo protein in *Drosophila* (plakoglobin) associated with E-cadherin. Science 254, 1359–1361.

McNeill H, Ryan TA, Smith SJ, Nelson WJ (1993). Spatial and temporal dissection of immediate and early events following cadherin-mediated epithelial cell adhesion. J Cell Biol 120, 1217–1226.

Montesano R, Matsumoto K, Nakamura T, Orci L (1991a). Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell 67, 901–908.

Montesano R, Schaller G, Orci L (1991b). Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. Cell 66, 697–711.

Nejsum LN, Nelson WJ (2007). A molecular mechanism directly linking E-cadherin adhesion to initiation of epithelial cell surface polarity. J Cell Biol 178, 323–335.

Nigam SK, Shah MM (2009). How does the ureteric bud branch? J Am Soc Nephrol 20, 1465–1469.

Nollet F, Kools P, van Roy F (2000). Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. J Mol Biol 299, 551–572.

O'Brien LE, Tang K, Kats ES, Schutz-Geschwender A, Lipschutz JH, Mostov KE (2004). ERK and MMPs sequentially regulate distinct stages of epithelial tubule development. Dev Cell 7, 21–32.

O'Brien LE, Yu W, Tang K, Jou TS, Zegers MM, Mostov KE (2006). Morphological and biochemical analysis of Rac1 in three-dimensional epithelial cell cultures. Methods Enzymol 406, 676–691.

O'Brien LE, Zegers MM, Mostov KE (2002). Opinion: building epithelial architecture: insights from three-dimensional culture models. Nat Rev Mol Cell Biol 3, 531–537.

Pirraglia C, Jattani R, Myat MM (2006). Rac function in epithelial tube morphogenesis. Dev Biol 290, 435–446.

Pollack A, Barth A, Altschuler Y, Nelson W, Mostov K (1997). Dynamics of beta-catenin interactions with APC protein regulate epithelial tubulogenesis. J Cell Biol 137, 1651–1662.

Pollack AL, Runyan RB, Mostov KE (1998). Morphogenetic mechanisms of epithelial tubulogenesis: MDCK cell polarity is transiently rearranged without loss of cell–cell contact during scatter factor/hepatocyte growth factor–induced tubulogenesis. Dev Biol 204, 64–79.

Qin Y, Capaldo C, Gumbiner BM, Macara IG (2005). The mammalian Scribble polarity protein regulates epithelial cell adhesion and migration through E-cadherin. J Cell Biol 171, 1061–1071.

Qin Y, Meisen WH, Hao Y, Macara IG (2010). Tuba, a Cdc42 GEF, is required for polarized spindle orientation during epithelial cyst formation. J Cell Biol 189, 661–669.

Rodriguez-Fraticelli AE, Vergarajauregui S, Eastburn DJ, Datta A, Alonso MA, Mostov K, Martin-Belmonte F (2010). The Cdc42 GEF Intersectin 2 controls mitotic spindle orientation to form the lumen during epithelial morphogenesis. J Cell Biol 189, 725–738.

Rosario M, Birchmeier W (2003). How to make tubes: signaling by the Met receptor tyrosine kinase. Trends Cell Biol 13, 328–335.

Santos OF, Barros EJ, Yang XM, Matsumoto K, Nakamura T, Park M, Nigam SK (1994). Involvement of hepatocyte growth factor in kidney development. Dev Biol 163, 525–529.

Schluter MA, Pfarr CS, Pieczynski J, Whiteman EL, Hurd TW, Fan S, Liu CJ, Margolis B (2009). Trafficking of Crumbs3 during cytokinesis is crucial for lumen formation. Mol Biol Cell 20, 4652–4663.

Stewart DB, Barth AI, Nelson WJ (2000). Differential regulation of endogenous cadherin expression in Madin-Darby canine kidney cells by cell–cell adhesion and activation of beta-catenin signaling. J Biol Chem 275, 20707–20716.

Takaishi K, Sasaki T, Kotani H, Nishioka H, Takai Y (1997). Regulation of cell–cell adhesion by rac and rho small G proteins in MDCK cells. J Cell Biol 139, 1047–1059.

Tinkle CL, Pasolli HA, Stokes N, Fuchs E (2008). New insights into cadherin function in epidermal sheet formation and maintenance of tissue integrity. Proc Natl Acad Sci USA 105, 15405–15410.

Troxell ML, Loftus DJ, Nelson WJ, Marrs JA (2001). Mutant cadherin affects epithelial morphogenesis and invasion, but not transformation. J Cell Sci 114, 1237–1246.

Tzaban S, Massol RH, Yen E, Hamman W, Frank SR, Lapierre LA, Hansen SH, Goldenring JR, Blumberg RS, Lencer WI (2009). The recycling and transcytotic pathways for IgG transport by FcRn are distinct and display an inherent polarity. J Cell Biol 185, 673–684.

Vega-Salas DE, Salas PJ, Gundersen D, Rodriguez-Boulan E (1987). Formation of the apical pole of epithelial (Madin-Darby canine kidney) cells: polarity of an apical protein is independent of tight junctions while segregation of a basolateral marker requires cell–cell interactions. J Cell Biol 104, 905–916.

Walker JL, Menko AS, Khalil S, Rebustini I, Hoffman MP, Kreidberg JA, Kukuruzinska MA (2008). Diverse roles of E-cadherin in the morphogenesis of the submandibular gland: insights into the formation of acinar and ductal structures. Dev Dyn 237, 3128–3141.

Wheelock MJ, Johnson KR (2003). Cadherins as modulators of cellular phenotype. Annu Rev Cell Dev Biol 19, 207–235.

Wu W, Kitamura S, Truong DM, Rieg T, Vallon V, Sakurai H, Bush KT, Vera DR, Ross RS, Nigam SK (2009). Beta1-integrin is required for kidney collecting duct morphogenesis and maintenance of renal function. Am J Physiol Renal Physiol 297, F210–F217.

Yaguchi S, Fukui Y, Koshimizu I, Yoshimi H, Matsuno T, Gouda H, Hirono S, Yamazaki K, Yamori T (2006). Antitumor activity of ZSTK474, a new phosphatidylinositol 3-kinase inhibitor. J Natl Cancer Inst 98, 545–556.

Yang J, Weinberg RA (2008). Epithelial–mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell 14, 818–829.

Yu W, O'Brien LE, Wang F, Bourne H, Mostov KE, Zegers MM (2003). Hepatocyte growth factor switches orientation of polarity and mode of movement during morphogenesis of multicellular epithelial structures. Mol Biol Cell 14, 748–763.

Zegers MM, Forget MA, Chernoff J, Mostov KE, ter Beest MB, Hansen SH (2003a). Pak1 and PIX regulate contact inhibition during epithelial wound healing. EMBO J 22, 4155–4165.

Zegers MM, O'Brien LE, Yu W, Datta A, Mostov KE (2003b). Epithelial polarity and tubulogenesis in vitro. Trends Cell Biol 13, 169–176.

Zhang X *et al.* (2009). beta1 integrin is necessary for ureteric bud branching morphogenesis and maintenance of collecting duct structural integrity. Development 136, 3357–3366.