Stable Knockdown of Polycystin-1 Confers Integrin-$\alpha 2\beta 1$–Mediated Anoikis Resistance

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The mechanisms of action of polycystin-1 (PC1) have been difficult to dissect because of its interaction with multiple factors, the heterogeneity of the genetic mutations, and the complexity of the experimental animal models. Here, stable knockdown of PC1 in MDCK epithelial cells was achieved by lentiviral-mediated delivery of a specific small interfering RNA for *PKD1*. The reduction of PC1 expression prevented tubulogenesis in three-dimensional collagen type I culture in response to hepatocyte growth factor and induced formation of cysts. PC1 knockdown created a condition of haploinsufficiency that led to hyperproliferation, increased adhesion to collagen type I, and increased apoptosis. It was shown that the suppression of PC1 was associated with the increased expression of integrin-$\alpha 2\beta 1$ and reduced apoptosis in cells grown on collagen type I. The engagement of integrin-$\alpha 2\beta 1$ seemed to be essential for the survival because PC1 knockdown cells were significantly less susceptible to anoikis by a mechanism that was reversible by anti–integrin-$\alpha 2\beta 1$ blocking antibodies. Overall, these data link integrin-$\alpha 2\beta 1$ to some of the biologic functions that are ascribed to PC1 and establish the potential of this approach for the direct study of PC1 functions in a genetically defined background. Furthermore, these findings indicate that reduction of PC1 expression levels, rather than the loss of heterozygosity, may be sufficient to induce cystogenesis.

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Alterations in the *PKD1* and *PKD2* genes account for cases of autosomal dominant polycystic kidney disease (ADPKD) with a relative frequency of approximately 85 and 15%, respectively (1–3). *PKD1* encodes polycystin-1 (PC1), a large 4302–amino acid membrane protein that participates in multiprotein complexes that are involved in cell–matrix and cell–cell interactions and signaling (4,5). Through the heterodimerization with PC2, a nonselective cationic channel that is encoded by the *PKD2* gene, PC1 can function as a gating receptor to control calcium influx (6). The interdependent functions of PC1 and PC2 in kidney are supported by the overlapping ADPKD phenotypic manifestation that is caused by mutations of either gene, although the severity and the progression of the disease are exacerbated when *PKD1* is involved. The co-localization of the polycystins in primary cilia suggests their involvement in a cellular mechanosensory system (7). These findings are supported by the evidence that polycystic renal phenotypes arise from the mutation or deletion of various ciliary and adhesion proteins (8–10). To date, however, it has been difficult to define clearly the functions of PC1 and the molecular mechanisms of cystogenesis that it controls. In part, this difficulty derives from the heterogeneity of the genetic mutations that are identified in patients with ADPKD as well as from the lack of functionally characterized mutants. Furthermore, most of the animal models for polycystic kidney share phenotypic manifestations, but some are genetically different or incompletely characterized.

Data concerning the functions of *PKD1* have been acquired from the study of knockout mouse models and from cells that have been isolated from patients with ADPKD. Although these sources have been extremely informative, they derive from the development and differentiation of genetically mutated cells, which may have evolved mechanisms compensating for PC1 dysregulation. As a result, some of the available small animal models do not parallel the progression of the disease that is observed in humans (11), and most have not been evaluated beyond the juvenile stage. Similarly, in studies that use PC1 transdominant mutants, it may be difficult to determine whether the ensuing effects are produced by the constitutive activation or the irreversible inhibition of PC1 normal functions. The specific inhibition of PC1 in characterized cell lines can facilitate the study of protein function by reproducing a condition of haploinsufficiency in the absence of other genetic variables.

In this study, constitutive knockdown of PC1 in MDCK cells was achieved by lentiviral-mediated expression of anti-PC1 siRNA. The stable expression of small interfering RNA (siRNA) that was specific for PKD1 inhibited the expression of PC1 and produced biologic features that are characteristic of the ADPKD phenotype, such as increased cellular adherence, cystic growth, and apoptosis. The apoptotic phenotype could be reverted by culturing the PC1 knockdown cells on type I collagen. Impor-

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tantly, we found that PC1 knockdown cells became resistant to anoikis through a mechanism that is mediated by integrin-$\alpha2\beta1$. These results suggest that anoikis resistance may play an important role in the expansion of the epithelia cells within the peritubular space and point at integrin-$\alpha2\beta1$ as a possible target to slow the cystogenic process. This novel approach circumvents possible compensatory developmental mechanisms, which cannot be excluded in cells that are derived from patients or animal models, and provides a unique tool for the genomic and proteomic comparative analyses in the presence or absence of functional PC1.

Materials and Methods

Cell Culture

Swiss 3T3 fibroblasts, MDCK-G (from hereon referred to as MDCK) and lentivirus-transduced cells lines were maintained at 37°C in a humidified, 5% CO_2 atmosphere in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Life Technologies) and 2 mM glutamine (Life Technologies). Conditioned medium from confluent Swiss 3T3 fibroblast monolayers was collected every 48 h, filtered through a 0.2-μm filter, and stored in aliquots at −80°C until used as source of hepatocyte growth factor (HGF) as previously reported (12,13).

Generation of the Lentiviral Vector for the Expression of siRNA

The vector VIRHD/E is a derivative of the VVCW self-inactivating lentivector (14). First, a NheI site was created by digestion with HindIII, fill-in with Klenow polymerase and self-ligation of the pPD31/C2 plasmid (15), which contains the murine phosphoglycerate kinase (PGK) promoter. The PGK promoter was excised from the resulting pPD31/CN plasmid with ClaI and NheI and ligated into the corresponding sites of VVCW, in place of the cytomegalovirus promoter, to generate the VVPW lentivector. The enhanced green fluorescence protein (EGFP) reporter then was amplified from the pEGFP1 plasmid (Clontech, Mountain View, CA) using the High Fidelity Taq polymerase (Roche Diagnostics, Indianapolis, IN) using the primers EGFP/SV40NLS/5 5′-ca*ggatcc*gccaccatgggcgacccaaaaaagaaaagaaaagtgagcaagggcgaggagctg-3′, which contains the SV40 nuclear localization signal, and EGFP/3 5′-gt*gaattc*ttacttgtacagctcgtccatgccgag-3′. The amplified product was digested with BamHI and EcoRI (italicized in primer sequences), filled in with Klenow polymerase, and ligated into the Klenow-filled in BamHI and NotI sites of the VVPW vector under the control of the PGK promoter to generate the VVWP/EGFP3 plasmid. A vector VVPW/E4 was generated from the insertion of unique XbaI and EcoRV restriction sites between the AscI and ClaI sites of VVPW/EGFP3 using a linker derived from the annealing of the following oligonucleotides that contained the XbaI (boldface) and EcoRV (italic) restriction sites: 5′-cgcgcc**tcta*ga***tatc*g-3′ and 5′-cgcgcc**tcta*ga***tatc*g. The synthetic human polymerase III H1 promoter then was generated by 10 cycles of PCR using High Fidelity Taq polymerase and the primers H1/A 5′-ggcgcgcc**gctagc**aatttgcatgtcgctatgtgttctgggaaatcaccataaacgtgaatgtctttggatttgggaat-3′ and H1/B 5′-gt**gctagc***gaattct*g*gatcc*gagtggtctcatacagaacttataagattcccaaatccaaagacatttcacgtttatgg-3′ that contained the NheI and BamHI and EcoRI cloning sites. The resulting 127-bp H1 product is flanked by NheI restriction sites (in bold in the primer) and provides the BamHI and EcoRI cloning sites (italicized in the primer) at the 3′ end of the promoter. After digestion with NheI, the H1 promoter was ligated into the XbaI site of VVPW/E4 to produce the VIRHC/E and VIRHD/E lentivectors. For the following study, we used the VIRHD/E lentiviral vector that contained the H1 promoter in the 3′-5′ orientation (Figure 1A). The following oligonucleotides were annealed and inserted between the BamHI and EcoRI sites of VIRHD/E so that specific siRNA were produced as short hairpin RNA under the control of the H1 promoter: PKD1/3211/S 5′-gatccg*ccacgtgagcaacgtcacc*attagatca*ggtgacgttgctcacgtgg*ttttttggaag-3′; PKD1/3211/AS 5′-aattcttccaaaaaa*ccacgtgagcaacgtcacc*tgatctaat*ggtgacgttgctcacgtgg*cg-3′; Luc/850/S 5′-gatcc*gtgcgttgctagtaccaac*ttcaagaga*gttggtactagcaacgcac*ttttttgg-3′; and Luc/850/AS 5′-aattccaaaaaa*gtgcgttgctagtaccaac*tctcttgaa*gttggtactagcaacgcac*g-3′ (specific complementary siRNA sequences are italicized).

Establishment of Cell Lines

Lentiviral vectors were produced and titered as described previously (14). MDCK cell were transduced at a multiplicity of infection of 20 in the presence of 5 μg/ml polybrene (Sigma, St. Louis, MO). One week after transduction, polyclonal cell populations that expressed the EGFP reporter protein were sorted using a triple-laser MoFlo high-speed cell sorter (Cytomation, Fort Collins, CO). Cells that were sorted at sheath pressure of 30 PSI and speed of 15,000 events/s then were expanded to prepare frozen batches. Individual clones from the control MDCK/E/siLuc and PC1 knockdown MDCK/E/siPKD cell populations were derived by limiting dilution.

Three-Dimensional Collagen Gels

MDCK and stably transduced cells were trypsinized to a single-cell suspension and resuspended at a concentration of 1×10^5 cells/ml in an ice-cold collagen solution that was prepared as described previously (13). Briefly, collagen type I solution (2.4 mg/ml; BD Biosciences, San Diego, CA) was mixed with 10× DMEM (Life Technologies) and 7.5% (vol/vol) bicarbonate solution (Life Technologies) in a ratio of 8:1:1. The cell suspension then was dispensed at 2.5 ml/well in six-well plates and allowed to gel for 20 min at 37°C before being overlaid with 2.5 ml of fibroblast-conditioned medium. The conditioned medium was changed daily, and pictures were taken at day 7.

Northern Blot Analysis

Total RNA was extracted from confluent monolayers using TRIzol (Invitrogen, Carlsbad, CA), and 20 μg from each sample was resolved by electrophoresis in 1.2% agarose gel. The RNA then was transferred onto nylon membrane and ultraviolet cross-linked. PC1 mRNA was detected using a 32P-labeled fragment that was amplified after reverse transcription of MDCK total RNA using the *PKD1*-specific primers PKDC-212/5 5′-gccaccgcgctagacgtc-3′ and PKDC-406/3 5′-cagaccgcagttacactcca-3′. The stripped membrane then was rehybridized with a probe specific for the housekeeping glyceraldehyde-3-phosphate dehydrogenase gene.

Immunodetection

Cells were lysed by incubation for 30 min on ice in lysis buffer that contained 1% Triton X100, 10 mM Pipes, 3 mM $MgCl_2$, 300 mM sucrose, and 5 mM EGTA and supplemented with a cocktail of protease inhibitors (Roche Diagnostics). For PC1 detection, 250 μg of each sample was resolved on a 4% SDS-polyacrylamide gel and electrophoretically blotted onto Immobilon-P membrane (Millipore, Bedford, MA). PC1 was detected using the affinity-purified goat anti-PC1 C-20 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 in PBS and 0.1% Tween-20 that contained 5% BSA and a secondary rabbit peroxidase-conjugated anti-goat antibody (1:5000 dilution in 5% nonfat dry milk in PBS and 0.1% Tween-20). Immunodetection of integrin-$\alpha2$ and integrin-$\beta1$ was performed on 100 μg of protein extracts resolved on 10% PAGE using a rabbit anti-human integrin-$\alpha2$ (Chemicon International, Temecula, CA) and anti-human integrin-$\beta1$–specific antibody

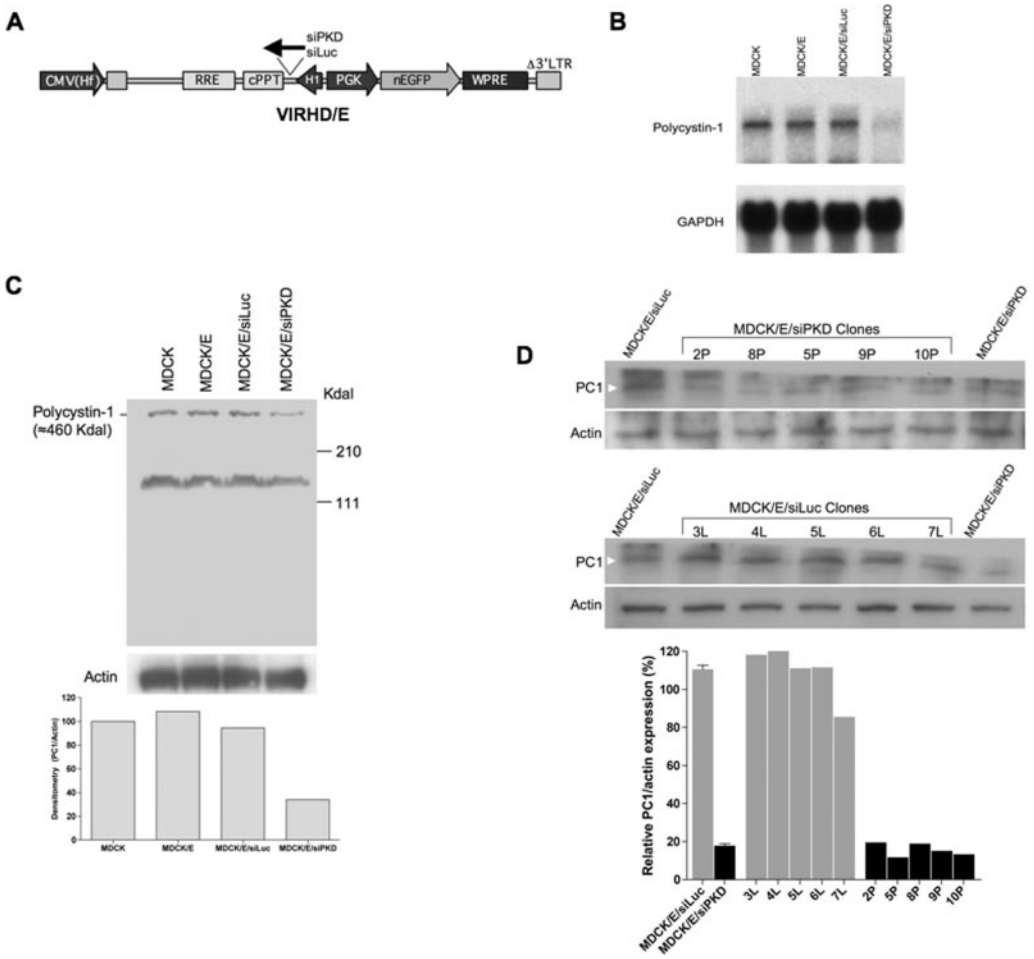

Figure 1. Knockdown of *PKD1* in MDCK cells. (A) Scheme of the VIRHD/E lentiviral vector and its derivatives VIRHD/E/siLuc and VIRHD/E/siPKD, which contain hairpin sequences for the expression of control anti-luciferase siRNA and anti-PKD1 siRNA, respectively. CMV, cytomegalovirus early promoter; RRE, rev responsive element; cPPT, central polypurine tract; H1, human H1 promoter; PGK, murine phosphoglycerate kinase promoter; nEGFP, enhanced green fluorescence protein with a nuclear localization signal; WPRE, Woodchuck hepatitis virus posttranscriptional regulatory element; Δ, deletion in 3′LTR. (B) Polycystin-1 (PC1) mRNA was detected by Northern analysis on total RNA that was isolated from the indicated cell populations using a probe that was specific for PKD1 (see Materials and Methods). The membrane then was stripped and rehybridized to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to control for equal sample loading (bottom). (C) Expression of PC1 was detected in total cell lysate from confluent cell cultures using the C20 antibody (Santa Cruz Biotechnology) specific for the PC1 carboxyl terminus. The position of molecular weight markers (Kdal) and PC1 are indicated. The approximately 150-Kdal band that was observed in all of the cell lines represents a nonspecific signal. The bars in the bottom panel represent the densitometric analysis that was performed on the radiographic film using the ImageJ software. The ratios of PC1 to actin expression are shown as percentage of that in parental MDCK cells. The expression of actin (indicated) was determined as control of equal loading after stripping of the membranes above and immunoblotting with an anti-actin antibody (dilution 1:20,000; Chemicon). (D) Expression of PC1 (white arrowhead) in individual clones that were derived from the control MDCK/E/siLuc (3L, 4L, 5L, 6L, and 7L) and PC1 knockdown MDCK/E/siPKD (2P, 5P, 8P, 9P, and 10P) polyclonal cell populations. Actin was used as loading control. The bars below the lanes indicate the densitometric analysis of the expression of PC1 relative to the correspondent actin signal in the control (▨) and PC1 knockdown (■) clones. MDCK/E/siLuc and MDCK/E/siPKD indicate the control and PC1 polyclonal populations, respectively.

(Upstate, NY) at a final dilution of 1:2000 and 1:5000, respectively. Actin expression was detected using a monoclonal mouse anti-actin antibody (Chemicon International) diluted 1:20,000. Immunocomplexes were visualized using the enhanced chemiluminescence detection kit Lumi-Light Plus (Roche Diagnostics).

FACS Analysis of Integrin Expression

For the detection of integrin-$\alpha2\beta1$ surface expression, 10^6 control MDCK/E/siLuc or PC1 knockdown MDCK/E/siPKD cells were harvested after incubation with dissociation buffer (Invitrogen). The cells then were washed once with medium that contained 10% FBS, twice in PBS that contained 0.1% BSA (PBS-0.1%BSA), and finally resuspended in 100 μl of PBS-0.1%BSA. The mAb specific for the integrin-$\alpha2\beta1$ heterodimer (clone BHA2.1; Chemicon International) (16,17) or isotype control antibody then was added to a final concentration of 10 μg/ml before the cells were incubated at 4°C for 30 min. The cells were washed twice in PBS-0.1%BSA and finally resuspended in 50 μl of the same buffer that contained 3 μg of CY3-conjugated goat anti-mouse IgG and

incubated at 4°C for 20 min. After three washes in PBS-0.1%BSA, red fluorescence was detected and quantified by flow cytometry.

Cell Adhesion Assay

Cells in single-cell suspension were plated in complete medium in a collagen-coated 96-well plate in sextuplicate at a concentration of 1×10^4 cells/well. After 1 h of incubation at 37°C, half of the wells were washed thrice with PBS before fresh medium was added to all of the wells. After a 24-h incubation, the number of remaining cells was determined using the Cell Titer 96 AQueous kit (Promega, Madison, WI). The cell count was normalized by expressing it as percentage of the number of originally plated cells in the unwashed wells.

Cell-Cycle Analysis

Parental and lentivector-transduced cells were trypsinized to a single-cell suspension, washed twice in PBS-0.1%BSA, and resuspended at the concentration of 1 to 2×10^6 cells/ml. Cells were fixed in 75% ethanol for 1 h at 4°C. After being washed twice in PBS, cells were incubated for 1 h at 4°C in 1 ml of staining solution that contained 50 μg/ml propidium iodide, 3.8 mM sodium citrate, and 500 ng/ml RNase A. The cells then were washed and analyzed by flow cytometry.

Apoptosis and Anoikis Assay

Apoptosis was determined by assessment of the expression of both annexin V and caspase-3 on cells that were plated as described in the text and figure legends. Annexin V was detected using the Vibrant Apoptosis Assay Kit # 9 (Molecular Probes, Eugene, OR), following the manufacturer's protocol, and then analyzed by flow cytometry. Caspase-3 was measured on equal amounts of cell extracts using the EnzChek Caspase-3 Assay Kit #1, (Molecular Probes) according to the manufacturer's instructions. For measurement of anoikis, cells were grown in nonadherent conditions using 100-mm Petri dishes that were coated with polyhydroxyethylmethacrylate (polyHEMA; Aldrich Chemical Co., Milwaukee, WI) as described (18). A total of 3,000,000 cells/ml were cultured for 12 h in regular tissue culture plates or in nonadherent conditions in polyHEMA-coated dishes before they were collected in the medium in which they had been incubated by scraping or pipetting, respectively. Floating cells in the tissue culture controls on uncoated plates were combined with the attached cells before protein extract preparation. The level of anoikis then was measured using the EnzChek Caspase-3 Assay Kit #1 (Molecular Probes). Blocking antibodies anti–integrin-$\alpha 2\beta 1$ were purchased from Chemicon and used at a final concentration of 10 μg/ml.

Statistical Analyses

Statistical analyses were performed by one-way ANOVA with Bonferroni post test or unpaired t test with Welch correction, using the InStat 3.0 software (GraphPad Software, San Diego, CA).

Results

Posttranscriptional Silencing of the PKD1 Gene

Silencing of PC1 expression was achieved by RNA interference. We used lentiviral vectors to stably express siRNA in the target cells to avoid possible variability of exogenously added siRNA and to allow the analysis of the suppression of PC1 during an extended period of time. MDCK cells were transduced with the control parental lentivector VIRHD/E and its derivative VIRHD/E/siLuc, which expresses a control anti-luciferase hairpin siRNA, or the VIRHD/E/siPKD lentivector, for the expression of a short hairpin sequence specific for PC1 (Figure 1A; see Materials and Methods for details). These vectors also carry the EGFP reporter gene whose expression was used to sort stably transduced polyclonal cell lines MDCK/E, MDCK/E/siLuc, and MDCK/E/siPKD, respectively. To assess the efficacy of the siRNA, the levels of PC1 mRNA were measured in transduced and nontransduced cells. Expression of PC1 mRNA was comparable in control MDCK, MDCK/E, and MDCK/E/siLuc cells, whereas it was dramatically decreased in MDCK/E/siPKD cells (Figure 1B). Immunodetection of PC1 using an anti-PC1–specific antibody indicated that the down-regulation of PC1 mRNA in the MDCK/E/siPKD cells corresponded to an approximately 75% decrease in the amount of PC1 protein (Figure 1C), confirming the efficiency of the anti-PC1 siRNA. The expression of PC1 also was determined in five randomly selected individual clonal populations that were derived from the control MDCK/E/siLuc (L clones) and PC1 knockdown MDCK/E/siPKD cells (P clones). Whereas it remained similar in the L clones, the expression of PC1 was reduced in all of the P cell lines, showing a degree of inhibition that ranged between 70 and 85% (Figure 1D). The average suppression of PC1 in the P clones confirmed the PC1 knockdown observed with the polyclonal populations. Therefore, unless differently specified, in the following experiments, the polyclonal populations were chosen because they were representative of different inhibition levels that may resemble more closely the *in vivo* expression distribution. The use of these populations also rules out clonal idiosyncratic responses.

Effects of PC1 Knockdown on In Vitro *Tubulocystogenesis*

We analyzed how the inhibition of PC1 expression would affect the structural organization of MDCK cells under these culture conditions. When grown in three-dimensional collagen gel culture in the absence or presence of exogenous HGF, MDCK cells have proved very useful as a model for cystogenesis or tubulogenesis, respectively. Within 3 d of culture in the presence of HGF, parental MDCK as well as control vector-transduced MDCK/E and MDCK/E/siLuc cells formed elongated cellular structures that acquired characteristic tubular shapes by day 7 (Figure 2A). In contrast, MDCK/E/siPKD cells failed to develop tubules and grew in small clusters that developed increasingly irregular cystic appearance with time (Figure 2A). Occasionally, some of the cellular formations in MDCK/E/siPKD were mixed structures with tubulocystic appearance (Figure 2A, bottom right). This observation suggested that fluctuations in the level of PC1 might be important to determine the final outcome, because it is expected that polyclonal cell populations exhibit different efficiency of PC1 mRNA inhibition and variable PC1 expression. A detailed histologic analysis was performed by sectioning the collagen-embedded cultures. The hematoxylin and eosin staining of preparations from MDCK/E/siPKD and control MDCK/E/siLuc cultures confirmed the cystic and tubular morphology, respectively (Figure 2B). Whereas tubules presented tight lumens, cysts were characterized by the typical flattened epithelium and larger, distended lumens. The relative numbers of tubular and cystic formations that were grown in the three-dimensional collagen cultures were scored under light microscopy (Figure 2C). A significantly higher number of tubular *versus* cystic structures was observed in paren-

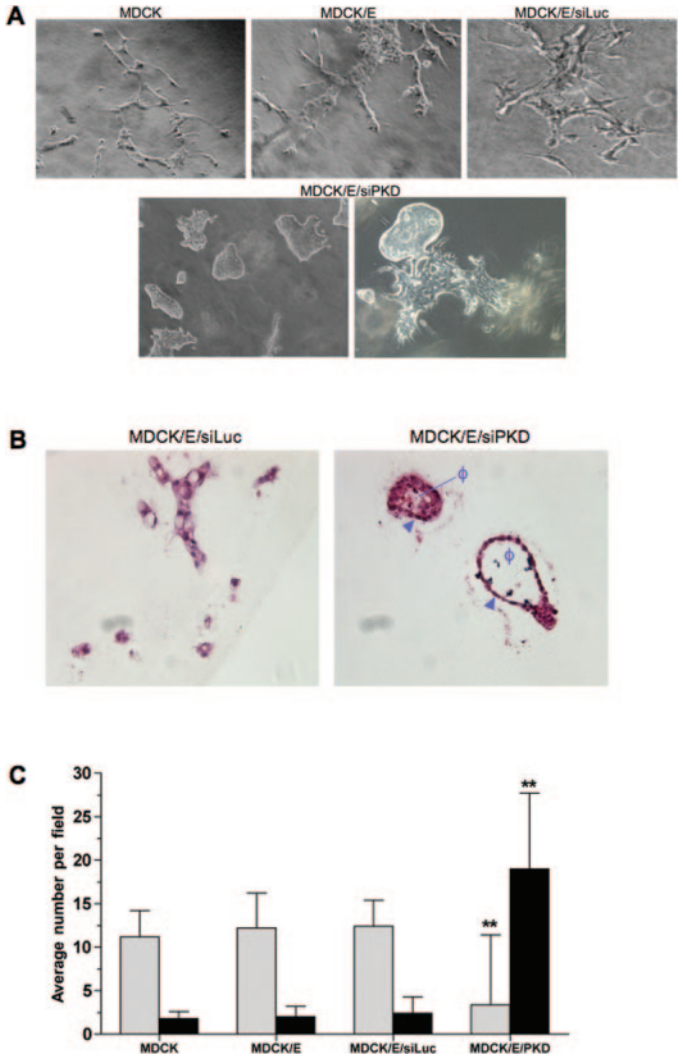

Figure 2. PC1 knockdown present a cystic phenotype of cells in three-dimensional collagen matrix culture. (A) Cells were grown in collagen type I gel in the presence of hepatocyte growth factor (HGF)-containing conditioned medium for 7 d before pictures were taken using a light microscope. Control cell lines MDCK, MDCK/E, and MDCK/E/siLuc produced extended tubule-like processes (top), whereas PC1 knockdown cells MDCK/E/siPKD presented irregular cystic-like structures (bottom). (B) Hematoxylin and eosin staining of the control MDCK/E/siLuc and PC1 knockdown MDCK/E/siPKD cultures grown in the collagen matrix gels. The luminal space (ϕ) and the flattened epithelial lining the cyst (arrowhead) are indicated. (C) The number of cysts *versus* tubules was scored in control and PC1 knockdown cells after 1 wk of culture in three-dimensional collagen gels in HGF. The bars indicate the average number of cysts and tubules ± SD for each cell line after evaluation of five random fields. $^{**}P < 0.01$ cyst or tubule count in PC1 knockdown cells *versus* controls. Original magnification, ×200.

tal MDCK and control MDCK/E or MDCK/E/siLuc cell cultures for 7 d. In contrast, in the MDCK/E/siPKD cultures, cystic structures significantly outnumbered tubules (Figure 2C).

Alteration of Cell Cycle and Apoptosis in PC1 Knockdown Cells

ADPKD is characterized by epithelial hyperproliferation, and overexpression of PC1 has been shown to inhibit cell growth (19). We assessed the growth rate in subconfluent cultures of control and PC1 knockdown cells. Suppression of PC1 expression correlated with significant increase of cell proliferation of MDCK/E/siPKD cells (Figure 3A). This results were supported further by the faster growth rate of PC1 knockdown clones as compared with the control clones (Figure 3B). Analysis of the cell cycle in nonsynchronized cultures in complete medium indicated a more pronounced decline of the fraction of PC1 knockdown cells in G1 by 72 h. At the same time, whereas in control MDCK/E/siLuc, the percentage of cells in the sub-G0/G1 fraction stabilized at approximately 4%, in PC1 knockdown MDCK/E/siPKD, the sub-G0/G1 fraction expanded to 16%, suggesting a higher rate of cell death (Figure 3C). The measurement of annexin V and caspase-3 levels confirmed that apoptosis was significantly increased in PC1 knockdown cells (Figure 4, A and C). It is interesting that growth on collagen type I–coated plates prevented the apoptosis that was associated with the inhibition of PC1 (Figure 4, B and D).

Increased Adherence and Expression of Integrin-α2 and Integrin-β1 in PC1 Knockdown Cells

The cystic phenotype is associated with multiple structural changes of the basement membrane and with abnormalities in the composition of matrix and cell–matrix interactions. ADPKD cells display increased adherence to type I collagen and increased levels of integrin-$\alpha2\beta1$ compared with normal age-matched human renal epithelia (20).

When we assessed the ability of PC1 knockdown cells to adhere to collagen type I, we measured a significantly increased attachment as compared with the parental or transduced control cells (Figure 5A, left). Similarly, PC1 knockdown clones were more adherent than control clones (Figure 5A, right). It is interesting that there seemed to be a correlation between the faster growth rate and the increased adherence within the PC1 knockdown clones and the relative PC1 expression (2P and 8P *versus* 5P, 9P, and 10P). Cell interactions with the extracellular matrix are mediated mainly by integrins. In particular, integrin-$\alpha2\beta1$, which functions as a receptor for collagen type I, has been shown to play a critical role in tubulocysts formation and the survival and the ability of renal tubular epithelia and MDCK cells to maintain polarization (21–23). To determine whether changes in integrin-$\alpha2$ and integrin-$\beta1$ expression may account for the differential adhesion to collagen type I, we measured the levels of these integrins in the various cell lines. Integrin-$\alpha2$ and integrin-$\beta1$ were equally expressed in MDCK, MDCK/E, and MDCK/E/siLuc. However, in the PC1 knockdown MDCK/E/siPKD cells, integrin-$\alpha2$ and integrin-$\beta1$ were increased three- and six-fold, respectively (Figure 5B). The higher relative expression of integrin-$\alpha2$ and integrin-$\beta1$ in the PC1 knockdown cells was maintained during culture in the presence of collagen type I (Figure 5B). FACS analysis using an antibody specific for the integrin-$\alpha2\beta1$ heterodimer showed a corresponding increase of integrin-$\alpha2\beta1$ on the surface of the MDCK/E/siPKD

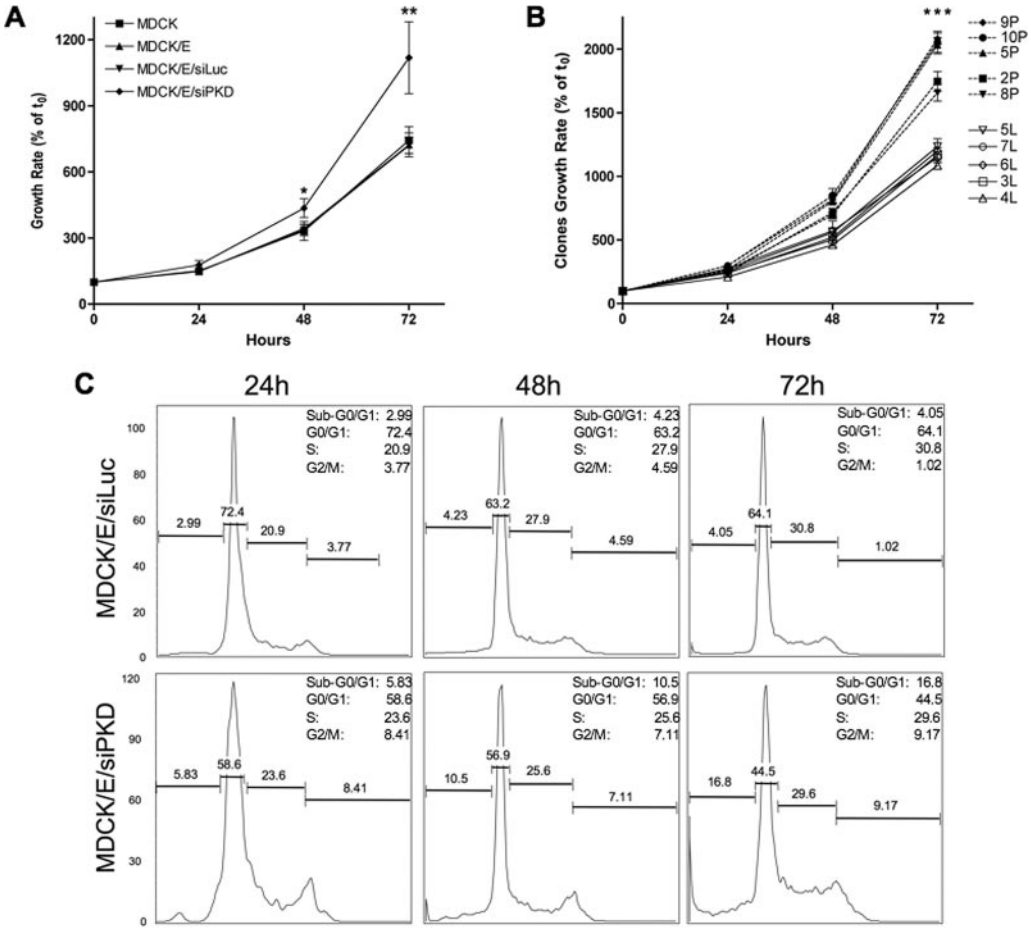

Figure 3. Increased proliferation and alteration of the cell cycle in PC1 knockdown cells. (A) Equal numbers of cells were plated and grown at subconfluent conditions for up to 72 h. At the indicated times, cells were harvested and counted. Cell counts are reported as percentage of the number of cells scored 2 h after plating (time 0). Each value represents the mean ± SD of three wells in one of five representative experiments. A significant increase in the number of the PC1 knockdown cells was detectable within 48 h and maximal at 72 h (*$P < 0.05$ and **$P < 0.01$, respectively, PC1 knockdown cells *versus* controls). (B) The same analysis that was performed on the clonal cell lines further indicated the faster growth rate of the PC1 knockdown cell lines. As expected, the clones display a narrower SD as compared with the polyclonal population and consequently a higher value of significance (***$P < 0.001$). (C) Control MDCK/E/siLuc and PC1 knockdown MDCK/E/siPKD cells were plated at subconfluent conditions and subjected to cell-cycle analysis at the indicated times of culture. PC1 knockdown MDCK/E/siPKD showed a decrease in the fraction of cells in G0/G1 by 72 h and a progressive increase in cellular death as compared with the control MDCK/E/siLuc culture.

cells as compared with the control cells, indicating the proper processing of the receptor (Figure 5C). This evidence, along with the prevention of apoptosis in PC1 knockdown cells that were plated on collagen type I (Figure 4, B and D), suggested that the engagement of integrin-$\alpha2\beta1$ by its ligand could provide a survival signal (24).

PC1 Knockdown Cells Are Resistant to Anoikis

The prevention of integrin-$\alpha2\beta1$ binding to collagen in MDCK cells was shown previously to induce anoikis, the mechanism of apoptosis that is triggered by the lack of cell adhesion to the matrix substrate (25–28). We observed that PC1 knockdown and control cells that were growing on collagen detached within 1 h from the addition of a blocking antibody that specifically interferes with the binding of the integrin-$\alpha2\beta1$ receptor to its substrates (Figure 6A). The cell detachment was followed by an increase in caspase-3 by 12 h in all of the cell lines, indicating the induction of anoikis. We therefore asked whether the augmented expression of integrin-$\alpha2\beta1$ in the PC1 knockdown cells may affect survival in conditions of anchorage-independent growth. When plated in nonadherent conditions, MDCK/E/siPKD seemed significantly more resistant to anoikis than the parental and control cells (Figure 6B). The direct role of integrin-$\alpha2\beta1$ in anoikis resistance in the MDCK/E/siPKD was assessed using the specific anti–integrin-$\alpha2\beta1$ antibodies. The presence of the anti–integrin-$\alpha2\beta1$ antibodies returned the level of anoikis in MDCK/E/siPKD to that observed in the parental or control transduced cells (Figure 6B), suggesting that the increase in integrin-$\alpha2\beta1$ is a determinant of the resistance to anoikis that is mediated by the integrin-$\alpha2\beta1$ heterodimer in PC1-deficient cells.

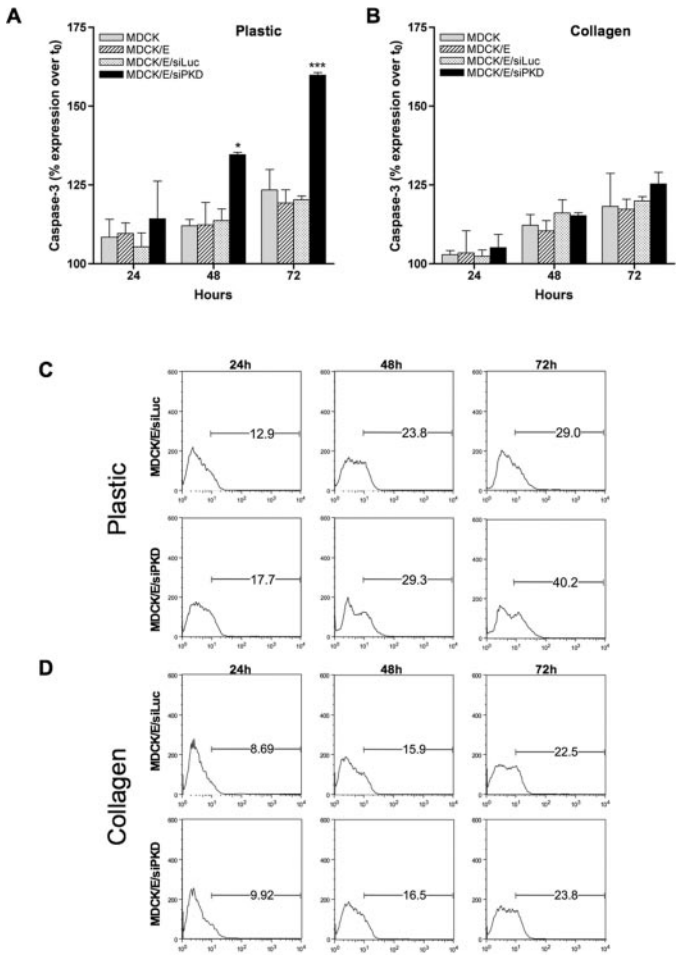

Figure 4. Apoptosis accounts for increased cell death in PC1 knockdown cells. An equal number of cells were cultured in adherent conditions on plastic (A and C) or collagen type I–coated plates (B and D) for 24, 48, or 72 h before being harvested to measure caspase-3 levels or annexin V. For each sample, the readings of caspase-3 adjusted for the relative protein concentration were expressed as percentage increase over the caspase-3 values of the respective cell population harvested after 2 h of culture (point 0). Shown is the mean ± SEM of three independent experiments. (A) Significant differences were observed between PC1 knockdown cells and controls (*$P < 0.05$; ***$P < 0.001$). (B) Apoptosis of PC1 knockdown cells was prevented when the cells were cultured on collagen type I as adhesion substrate. (C and D) Annexin V, measured by FACS analysis, confirmed the highly apoptotic phenotype of PC1 knockdown MDCK/E/siPKD cells as compared with the control MDCK/E/siLuc (C) and its reversal after plating on collagen type I (D).

Discussion

To analyze directly the biologic functions of PC1, we used siRNA interference to suppress the expression of PC1 in MDCK cells, which provide a useful model for studying the mechanisms of tubulocystogenesis. We showed that lentiviral-mediated delivery of anti-PC1–specific siRNA efficiently suppresses PC1 expression in MDCK cells and induces a state of haploinsufficiency that recapitulates the major biologic alterations that are pathognomic to ADPKD. Specifically, PC1 knockdown MDCK lost the ability to form tubules when grown in three-dimensional collagen gel in the presence of HGF. Similar to observations in human renal ADPKD cyst epithelial cells, PC1 knockdown cells were more adherent to collagen type I and grew at a faster rate than the controls, while maintaining a higher apoptotic phenotype (4,29). These data support the findings of Boletta *et al.* (17) showing slow growth, inhibition of apoptosis, and induction of tubule formation in MDCK cells that overexpressed PC1.

We used the siRNA knockdown model to show that the specific suppression of PC1 leads to stronger adherence and increased expression of integrin-α2 and integrin-β1, whose heterodimer recognizes collagen type I as ligand. These observations complement those by Wilson *et al.* (20), who reported enhanced integrin-α2 expression in cell lines that were established from patients with ADPKD and inhibition of adherence to collagen type I by anti–integrin-$\alpha2\beta1$ antibodies. Increased adherence to collagen type I, possibly mediated by integrin-β1, also was observed in cell lines that were derived from the C57Bl/6J*cpk* mouse model of autosomal recessive polycystic kidney disease (ARPKD), which carries a truncation in the cystin gene (29,30). Taken together, these findings emphasize the importance of integrin-β1 in polycystic kidney disease independent of the genetic basis underlying the cystogenic process. In addition, we demonstrated that growth in the presence of collagen type I reverts the apoptotic phenotype of PC1 knockdown cells, suggesting that a positive feedback may potentiate the integrin-mediated survival signal when PC1 is absent or expressed at low levels. These results are consistent with the finding that suppression of integrin-$\alpha2\beta1$ leads to increased cell death of MDCK and fails to branch in response to HGF or to form cysts in collagen type I (22). Although integrins are involved mostly in the binding to extracellular matrix, they also play a role in cell–cell contact (31). In particular, integrin-β1 has been shown to bind E-cadherin in a divalent cation-dependent manner (32). The colocalization of integrin-$\alpha2\beta1$ with PC1 at the focal adherens (20) and the presence of E-cadherin in the multiprotein complex with PC1 (33) suggest that interactions among these molecules may modulate cell–cell and/or cell–matrix adhesion during epithelial remodeling. Recently, the re-expression of N-cadherin along with a reduction of E-cadherin has been demonstrated on the membranes of cyst-lining epithelial cells from patients with ADPKD (34), an effect that is consistent with the epithelial-mesenchymal transition and loss of polarization and dedifferentiation of cystic epithelial cells (4). Whether N-cadherin can interact with integrin-$\alpha2\beta1$ and provide a survival signal remains to be established. However, because integrin-β1 has been localized in the cilium, where it participates in the flow-induced Ca^{2+} response (35), it remains possible that its modulation plays a role in the ciliary mechanosensory function of polycystins.

Our data also showed for the first time that PC1 knockdown cells seem more resistant to anoikis—the apoptotic pathway that is triggered by the loss of cell anchorage—through a mechanism that involves integrin-$\alpha2\beta1$. It is possible that integrin-$\alpha2\beta1$–mediated cytoskeletal rearrangement and other changes at the membrane of PC1 knockdown cells allow different surface molecules to interact and transmit survival cues and favor the expansion of the cystic epithelium into the renal interstitium. Changes in the extracellular

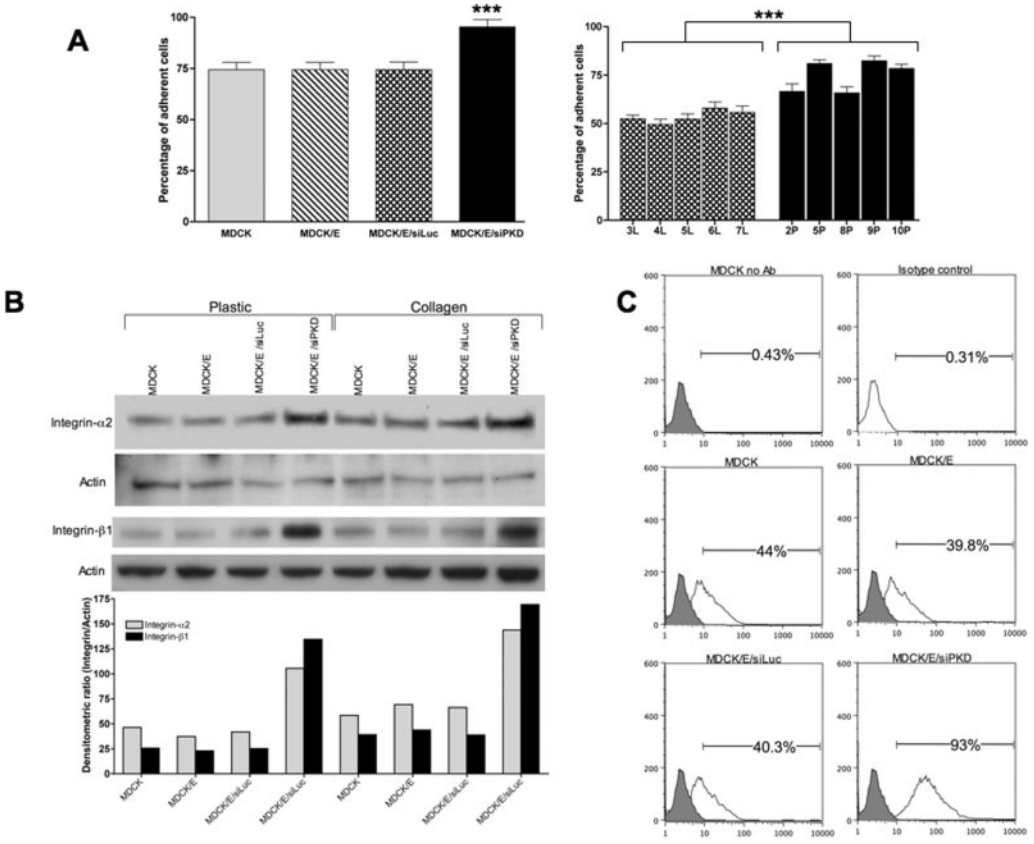

Figure 5. PC1 knockdown cells display increased adherence to collagen type I and higher expression of integrin-α2 and integrin-β1. (A, left panel) Single-cell suspensions of control and PC1 knockdown cells were plated on collagen type I–precoated plates for 1 h. Cells then were washed three times with PBS before fresh medium was added. The number of remaining adherent cells, normalized by the number of originally plated cells (unwashed), was determined using the Cell Titer 96 AQueous kit (Promega). Data are means ± SD of sextuplicate counts (***$P < 0.001$) in one of five independent experiments. (A, right panel) Adhesion experiments on individual clones confirmed the highly significant increase of adherence of PC1 knockdown P (P2, P5, P8, P9, and P10) cell lines as compared with control L (3L, 4L, 5L, 6L, and 7L) clones ($P < 0.0001$, unpaired t test with Welch correction performed on aggregate values from individual clones). (B) Cells were plated either on uncoated or collagen type I–precoated plates for 24 h, as indicated. Integrin-α2 and integrin-β1 expression was detected by immunoblot. Actin expression then was determined on the stripped membrane to control for equal sample loading. The expression of integrin-α2 and integrin-β1 relative to actin was quantified using ImageJ software (NIH) and depicted in the bar graph below the immunoblot. The increased expression of either integrin was not significantly affected by culturing on collagen type I. (C) Surface expression of integrin-α2β1. FACS analysis that was performed using an anti–integrin-α2β1–specific antibody indicated that the heterodimer was more highly expressed on the plasma membrane of PC1 knockdown MDCK/E/siPKD as compared with control MDCK/E/siLuc cells.

matrix composition are likely to contribute to these interactions and modulate the cellular architecture. In fact, in agreement with previous observations (36,37), the culture of both control and PC1 knockdown cells in basement membrane matrigel failed to develop tubules and grew as cysts regardless of the presence of HGF (data not shown).

It is interesting that both the increase in integrin expression and reduced anoikis are characteristic of the metastatic evolution of tumors (38). Similarly, N-cadherin has been involved in the progression of the metastatic phenotype (39). Although we did not assess N-cadherin expression, the overexpression of integrin-β1, hyperproliferation, and resistance to anoikis after the inhibition of PC1 are suggestive of the emergence of a preneoplastic profile and support the proposed role of PC1 as a tumor suppressor gene (19). To date, no correlation between ADPKD and cancer has been established, but the occurrence of renal carcinoma after acquired polycystic kidney disease secondary to hemodialysis is well documented (40). How these two cystogenic processes differ remains an intriguing question, and the definition of the variables and the conditions that lead to transforming events in acquired polycystic kidney but not in ADPKD or ARPKD may reveal new functions of PC1.

PC1 knockdown also inhibited tubulogenesis of murine inner medullary collecting duct cells in three-dimensional collagen culture (L.B. and G.L.G., data not shown). Despite that similar effects are observable in various species, the use of culture-adapted cell lines, which may carry unknown genetic alterations, prevents firm conclusions on the contribution of other mutational mechanisms to the onset of ADPKD. However, the specificity of PC1 suppression by siRNA strongly suggests that the levels of PC1 expression

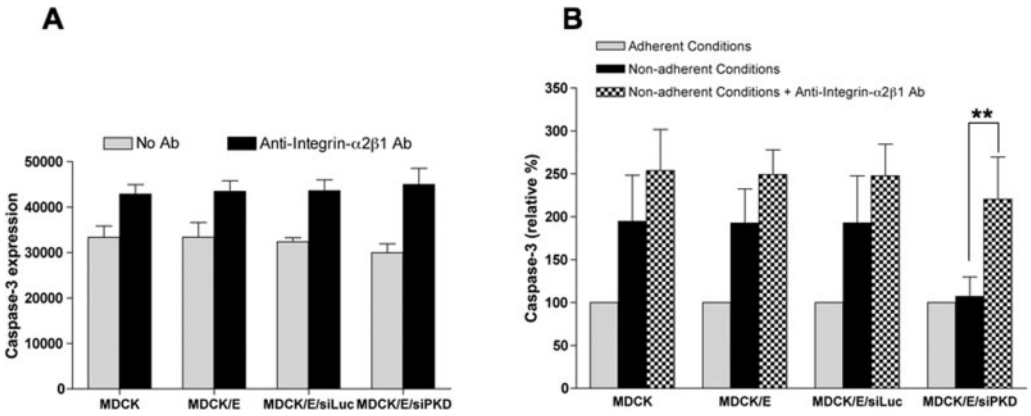

Figure 6. PC1 knockdown cells are resistant to anoikis by a mechanism that is mediated by integrin-$\alpha2\beta1$. (A) Control and PC1 knockdown cells were grown on collagen type I–coated plates for 24 h and then incubated in the absence or the presence of the blocking antibody specific for the integrin-$\alpha2\beta1$ heterodimer. The presence of the antibody equally induced cell detachment (data not shown) and caspase-3 activation in control and PC1 knockdown cells. (B) Equal numbers of control and PC1 knockdown cells were plated either under adherent conditions on tissue culture dishes or under nonadherent conditions on polyhydroxyethylmethacrylate-coated Petri dishes in the absence or presence of blocking anti–integrin-$\alpha2\beta1$ antibodies (Chemicon; see Materials and Methods). Anoikis was determined by measurement of the levels of caspase-3 in nonadherent conditions expressed as a percentage of the relative caspase-3 values in adherent conditions. Data are means ± SD. Significant change in anoikis as a result of the co-incubation with the anti–integrin-$\alpha2\beta1$ antibodies (checkered bars) was observed in PC1 knockdown cells (**$P < 0.01$ *versus* PC1 knockdown cells under nonadherent condition in the absence of anti–integrin-$\alpha2\beta1$ antibodies).

play a fundamental role in the cystogenic process. Overall, our *in vitro* findings support the haploinsufficiency model for ADPKD development in agreement with the observation of cyst formation in *PKD1* null heterozygotes (41) and a *PKD1* hypomorphic mouse (42). Because the overexpression of PKD1 produces a cystic phenotype (43,44), it seems that PC1 needs to be expressed within a specific range to maintain normal renal tubule dimensions. These data are consistent with the notion that fluctuations of the protein levels above or below the normal thresholds will unbalance its stoichiometric relationship with other interacting factors leading to the cystogenic signal. These changes likely occur stochastically and may require the contribution of other genetic and/or environmental events to establish the cystogenic process. Such a mechanism may explain the low frequency of cystic nephrons and the different onset and variable course of the disease within members of a family who carry the same germline mutation. Our results further indicate that the stable siRNA-mediated PC1 knockdown represents an important complement to genetic studies and may be uniquely suited for the comparative genomic and proteomic analyses to identify cystogenic pathways that are triggered by the fluctuation in the expression of PC1.

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