Thiazolidinediones inhibit MDCK cyst growth through disrupting oriented cell division and apicobasal polarity

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Mao Z, Streets AJ, Ong AC. Thiazolidinediones inhibit MDCK cyst growth through disrupting oriented cell division and apicobasal polarity. Am J Physiol Renal Physiol 300: F1375–F1384, 2011. First published March 23, 2011; doi:10.1152/ajprenal.00482.2010.—Thiazolidinediones have been reported to retard cystic disease in rodent models by uncertain mechanisms. We hypothesized that their major effect in retarding cystogenesis was through inhibiting cell proliferation or stimulating apoptosis. In the Madin-Darby canine kidney cell (MDCK) model, rosiglitazone inhibited cyst growth in a time- and dose-dependent manner and this was accompanied by a reduction in basal proliferation and an increase in apoptosis. Unexpectedly, we also observed a striking abnormality in lumen formation resulting in a characteristic multiple lumen or loss of lumen phenotype in treated cells at doses which did not inhibit cell proliferation. These changes were preceded by mislocalization of gp135 and Cdc42, misorientation of the mitotic spindle, and retardation in centrosome reorientation with later changes in primary cilia length and mislocalization of E-cadherin. Cdc42 activation was unaffected by rosiglitazone in monolayer culture but was profoundly inhibited in three-dimensional culture. MDCK cells stably expressing mutant Cdc42 showed a similar mislocalization of gp135 expression and multilumen phenotype in the absence of rosiglitazone. We conclude that rosiglitazone influences MDCK cyst growth by multiple mechanisms involving dosage-dependent effects on proliferation, spindle orientation, centrosome migration, and lumen formation. Correct spatial Cdc42 activation is critical for lumen formation, but the effect of rosiglitazone is likely to involve both Cdc42 and non-Cdc42 pathways.

PPARγ; polarity; Cdc42; lumen; PKD

AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE (ADPKD) is the most common inherited renal disease with a reported incidence of 1 in 1,000 live births. It is the fourth most common cause for end-stage renal failure. ADPKD is caused by mutations in two genes, PKD1 (85%) and PKD2 (15%), and is characterized by the formation and expansion of fluid-filled cysts which eventually destroyed kidney structure and function (23).

The pathogenesis of cyst formation in ADPKD has been intensively investigated, and several potential mechanisms suggested by clinical and experimental studies. These include increased cell proliferation and apoptosis, enhanced fluid secretion, abnormal cell-matrix interactions, alterations in cell polarity, and abnormalities in ciliary structure or function (5).

At present, there are no effective treatments for ADPKD in humans although a number of compounds have been found to work in rodent PKD models (5). Among these are peroxisome proliferator-activated receptor-γ (PPARγ) agonists or thiazolidinediones (TZDs). TZDs, including rosiglitazone and pioglitazone, are synthetic ligands for the PPARγ receptor and are in widespread clinical use for the treatment of type 2 diabetes due to their insulin-sensitizing properties. It has also been recognized that TZDs also have multiple anti-inflammatory, anti-fibrotic, and vascular effects independent of blood glucose lowering (17). Of relevance to PKD, maternal administration of pioglitazone improved the postnatal survival of Pkd1 null mouse embryos, and this was associated with a reduction in renal cystic disease by uncertain mechanisms (21). More recent studies have shown a survival benefit in conditional (principal cell) Pkd1 null mice associated with a reduction in systemic blood pressure (but without an effect on cyst formation) and Han:SPRD rats, with a moderate effect on kidney and liver cyst volumes in female Pck rats (2, 6, 25). Nonetheless, the mechanism of these reported effects remains unclear, with one study reporting no effect on cell proliferation or apoptosis, another reporting reductions in the expression of profibrotic [transforming growth factor (TGF)-β1] and inflammatory (MCP-1) cytokines and Wnt (β-catenin) signaling, and finally decreased CFTR expression by cyst epithelia (and thus Cl− secretion) (2, 6, 25).

Recently, long-term cardiac safety concerns have been raised for TZDs which are clinically significant given their widespread use. In view of the potential beneficial effects of TZDs in ADPKD, a useful approach would be to clarify their underlying therapeutic mechanism on cyst expansion so as to minimize off-target effects. In this study, we sought to investigate the potential mechanisms of PPARγ activation on cystogenesis using the well-established three-dimensional (3D) Madin-Darby canine kidney (MDCK) model.

MATERIALS AND METHODS

Materials and antibodies. All chemicals were purchased from Sigma (Poole, UK) unless otherwise stated. Alexa 594-phalloidin was purchased from Invitrogen (Carlsbad, CA). The gp135 mAb was a gift of G. Ojakian (SUNY, New York). Antibodies against E-cadherin and calnexin were purchased from BD Transduction Labs (Oxford, UK), cdc42 from Santa Cruz Biotechnology (Santa Cruz, CA), γ-tubulin and acetylated α-tubulin from Sigma-Aldrich, Ki-67 from Dianova (Hamburg, Germany), and cleaved caspase-3 (Cell Signalling Technology, NEB, Hitchin, Hertfordshire, UK). Goat anti-mouse FITC and rabbit anti-mouse IgG (H+L) FITC were from Southern Biotech, (Birmingham, AL), and donkey anti-goat IgG (H+L) Alexa Fluor 568 was from Invitrogen. The GST-CRIB-PAK1 plasmid (34) was obtained from A. Rajasekaran (gift of Dr. Y Zheng). Parental MDCK II (T23) and stable transfectants expressing tetracycline-inducible myc-tagged dominant-negative Cdc42 (Cdc42N17) or constitutively active...
Cdc42 (Cdc42V12) were a gift of W. J. Nelson (Stanford, CA; obtained through J. H. Lipschutz) (27).

**Cell culture.** MDCK II cells were cultured in DMEM (GIBCO) with a supplement of 1% antibiotic/antimycotic (AA, GIBCO), 1% L-glutamine, and 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂, and the medium was changed every 2 days.

Cytotoxicity assays. LDH release and crystal violet assays were used to establish the therapeutic/toxic dose range for rosiglitazone. For the LDH release assay, a Cytotoxicity Detection KitPLUS LDH (Roche) was used according to the manufacturer's instructions.

The crystal violet assay was performed as previously described (13). Briefly, MDCK cells were incubated for 24 h on 96-well plates and then incubated for 24 h with rosiglitazone (from 0.05 to 1,000 μM). DMSO treatment was set as a control. The medium was removed, and adherent cells were fixed with 100 μl/well ice-cold methanol/acetone (1:1 vol) and stained for 60 min with 0.2% crystal violet in 2% methanol. Plates were washed with distilled water and air dried thoroughly. Stain was extracted with 100 μl/well 10% acetic acid at room temperature with shaking for 30 min, and absorbance was measured at 570 nm.

Cystogenesis assays. Cystogenesis assays were performed as previously described with minor modifications (28). Briefly, MDCK II cells were detached with trypsin/EDTA (Invitrogen) and pipetted into ice-cold type I collagen-based medium (70% vol/vol, 3.84 mg/ml collagen I, 20% 11.76 mg/ml NaHCO₃ solution and 10% 10× MEM) to make a single-cell suspension at a final plating density of 1 × 10⁴ cells/ml. The collagen-based medium with cells was aliquoted into 96-well plates at 100 μl/well, and the plates were incubated at 37°C for 20 min to promote gelation. Two hundred microliters of medium was introduced into each well after gelation. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂, and the medium was changed every 2 days.

To induce Cdc42 expression in MDCK lines expressing Cdc42N17 or Cdc42V12, tetracycline was first removed, and the cells were changed every 2 days. For the LDH release assay, a Cytotoxicity Detection KitPLUS LDH (Roche) was used according to the manufacturer's instructions. The crystal violet assay was performed as previously described (13). Briefly, MDCK cells were incubated for 24 h on 96-well plates and then incubated for 24 h with rosiglitazone (from 0.05 to 1,000 μM). DMSO treatment was set as a control. The medium was removed, and adherent cells were fixed with 100 μl/well ice-cold methanol/acetone (1:1 vol) and stained for 60 min with 0.2% crystal violet in 2% methanol. Plates were washed with distilled water and air dried thoroughly. Stain was extracted with 100 μl/well 10% acetic acid at room temperature with shaking for 30 min, and absorbance was measured at 570 nm.

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To induce Cdc42 expression in MDCK lines expressing Cdc42N17 or Cdc42V12, tetracycline was first removed, and the cells were treated with rosiglitazone (20 μM) or DMSO 24 h after plating into collagen and cultured for 7 days.

To monitor the growth rate of individual cysts, each cell was identified with a unique reference number relative to a grid placed under the plate after seeding (32). Phase-contrast images of cells were taken by an inverted microscopy (Olympus IX71) from day 2 and followed every 2 days. At least 20 cysts/group were followed, and the cyst section with the widest transverse diameter was chosen for image analysis. The overall transverse area of each cellular structure at different time points was measured using ImageJ [National Institutes of Health (NIH)]. The growth rate of each cellular structure was calculated as (change of cyst area between two time points/cyst area at former time point) × 100%.

For the end point assay, gels were treated in triplicate with rosiglitazone (20 μM) or DMSO for 24 h. After washing with PBS twice, cells were fixed with 4% PFA and stained with antibody to cleaved caspase-3 (1:100) and DAPI. Cleaved caspase-3-positive cells were counted in 50 random fields (>600) without overlap in each group, and the results were adjusted by the total cell numbers in each field. Similarly, MDCK cells in collagen gels treated with rosiglitazone (20 μM) or DMSO before (day 3) or after (day 6) lumen formation were fixed with 4% PFA and stained with an antibody to cleaved caspase-3 (1:100). To quantify the degree of apoptosis, 50 random fields (>400) without overlap were selected in each group, and the percentage of cysts with cleaved caspase-3-positive cells were calculated.

To assess the location of caspase-3-positive cells in each group, co-staining with gp135 was performed and DIC images were simultaneously captured. Caspase-3-positive cells were considered to be “luminal” if they were clearly located in the central cyst lumen in both gp135 and DIC images. The number of cysts with luminal apoptotic cells were quantified and expressed as a percentage of the total number of cysts with apoptotic cells counted.

**Measurement of mitotic spindle angles.** To increase the number of cells undergoing mitosis, a thymidine block was introduced as previously described with modification for 3D culture (10). Briefly, MDCK cells in collagen gels were cultured in complete medium for 72 h to form microcysts. They were then incubated with medium containing 3 mM thymidine (Sigma) for 48 h to arrest cells at the early S phase and simultaneously treated with rosiglitazone (20 μM) or DMSO. To release cells from the S phase, the gels were extensively washed with PBS (6 × 10-min washes) to remove thymidine. They were then further incubated with medium containing rosiglitazone or DMSO for another 24 h to permit entry into the late G2 phase.

Gels were fixed with 4% PFA and stained with α-tubulin antibody (1:800) and phalloidin to visualize the mitotic spindle and actin cytoskeleton. To measure the spindle angles, z-stack images of cells undergoing metaphase and early anaphase cells were captured in the
middle region of the cysts and merged to ensure that both poles of a
spindle were in the same image. The centroid was defined as the point
of intersection between lines representing the longest and shortest
diameters of the cyst. The center of the spindle axis was defined as the
midpoint of a line connecting the two poles of the spindle. The angle
between the spindle axis and the line connecting the centroid of the
cyst and the center of the spindle axis was analyzed, as shown in Fig.
5C (12). Cells were defined as being in metaphase when chromosomes
were aligned at the cell equator. Early anaphase was defined as the
point at which sister chromatids had just started to separate, but cell
alignment and membrane invagination had not yet occurred.

Reorientation of centrosomes. To assess the reorientation of cen-
trosomes, cells treated with rosiglitazone or control were seeded on
collagen I-coated coverslips in six-well plates and incubated with
complete medium for 4, 24, or 72 h. The seeding cell density was
chosen to ensure that cells would reach complete confluence but not
be overconfluent at 4 (100,000 cells/ml), 24 (50,000 cells/ml), or 72-h
(30 × 10^4 cells/ml) experiments. Cells were then fixed with ice-cold
100% methanol and stained for γ-tubulin (antibody dilution 1:800)
and DAPI.

Z-stack images of centrosomes stained with γ-tubulin were ob-
tained with a z-axis thickness of 0.1 μm. The distance between the
centrosomes and basal membrane was measured to assess the reori-
entation rate of centrosomes to the apical side of the nucleus (8).

Measurement of cilia length and number. Measurement of cilia
length was modified from a previous report (31). In brief, MDCK
cysts were fixed on days 12, 14, and 17 and stained with α-acetylated
tubulin (antibody dilution 1:800) and phalloidin to visualize the
primary cilia and cystoskeleton. Thirty cysts were randomly selected
from each group, and images of cysts were taken at different focal
planes to visualize clearly the entire cilia length.

To measure cilia number and length in monolayer culture, cells
were seeded on collagen-coated coverslips, cultured for 7 days, then
fixed and stained for acetylated-α-tubulin and DAPI. Cilia lengths and
numbers were measured and counted with Image J, and the number of
cilia was normalized to the number of nuclei for both groups.

Cdc42 activation assay. MDCK cells on monolayer culture were
rinsed with ice-cold PBS after a 24-h incubation with rosiglitazone or
DMSO, and total cell lysates were prepared on ice and clarified by
ultracentrifugation. Fifty microliters of cell supernatant was set aside
for determination of total cdc42 and protein concentrations, and the
rest was used to determine GTP-bound Cdc42 using a pull-down
assay (see below). For cyst assays, cells were plated in collagen gels
at a density of 4 × 10^4 cells/ml, treated with rosiglitazone (20 μM) or
DMSO 24 h after plating, and cultured for a further 6 days. Cyst-
containing collagen gels were incubated in calcium-free HBSS con-
taining 4,000 U/ml collagenase, 0.005% DNase, and protease inhib-
itor cocktail for 45 min at 37°C with gentle rotation to digest the
collagen matrix. Intact cysts were then pelleted from the collagenase-
treated mixture by centrifugation at 3,000 g for 3 min, washed twice
with PBS, and then lysed in IP buffer.

To detect active or GTP-bound Cdc42, a GST pull-down assay
using a GST-PAK fusion protein (which binds to GTP-Cdc42 but not
GDP-Cdc42) was performed as previously described (9). In brief, 2
μg of GST (control) or GST-PAK protein was added to 500 μg of
DMSO or rosiglitazone-treated cyst cell lysate, and the samples were
incubated with rotation for 1 h at 4°C. The GST protein was pulled
down by incubation with glutathione 4B Sepharose beads for 2 h at
4°C. Beads were washed three times with PBS and boiled for 5 min
in 40 μl SDS-PAGE loading buffer before loading on the gel. GTPγS
(100 μM) or GDP (1 mM) loading of cell lysates in vitro were used
as positive and negative controls. In vivo stimulation will activate
~10% of the available Cdc42, whereas in vitro GTPγS protein
loading will activate nearly 100% of available Cdc42. Equal concen-
trations of lysates were added to two tubes, and EDTA was added to
a final concentration of 10 mM. Samples were then incubated at 30°C

Fig. 1. Growth rate of Madin-Darby canine kid-
ney cell (MDCK) II cysts was reduced by rosigli-
tazone (Rosi). Individual MDCK cysts were mon-
tored every 2 days after plating by phase-contrast
microscopy, and the cyst area was measured se-
quentially. A: the average area of MDCK II cysts
was significantly reduced by rosiglitazone (20
μM). Values are means ± SE (n = 20–27). B: ex-
ample of an MDCK II cyst treated with
DMSO (n = 20) or rosiglitazone 20 μM (n = 27)
monitored serially from day 0 to day 12. Scale
bar = 100 μm. *P < 0.05, **P < 0.01 vs.
DMSO.
for 15 min with agitation, and a GST pull-down was carried out with the GST-PAK as described above.

Samples were blotted with anti-Cdc42 or anti-GST antibodies. Total Cdc42 was shown to be equal in both DMSO- and rosiglitazone-treated cells following loading of equal concentrations of cell lysate (20 μg/lane).

Statistical analysis. All data are expressed as means ± SE. Student’s unpaired t-test was applied with GraphPad Prism software.

RESULTS

Rosiglitazone inhibits the overall growth of MDCK cysts. Preliminary assays showed that rosiglitazone had no effect on cell viability (crystal violet) or toxicity (LDH release) at doses of up to 100 μM in monolayer culture (not shown). Sequential analysis of cyst area from days 2–12 showed that 20 μM rosiglitazone significantly inhibited MDCK cyst growth from day 6 compared with DMSO-treated controls (Fig. 1, A and B).

Rosiglitazone inhibits proliferation and induces apoptosis of MDCK cysts. Cell counts in MDCK cysts after 13-day incubation showed a significant dose-dependent decrease above 10 μM (Fig. 2A). Since this reduction could be the consequence of a decrease in proliferation and/or increase in apoptosis, we first investigated cell proliferation in MDCK cysts using the proliferation marker Ki-67. Rosiglitazone treatment (20 μM, 8 days) decreased the number of Ki-67 positive cells per cyst by 70% (from 47.9 ± 2.6 to 14.5 ± 1.0 positive cells/cyst, P < 0.0001, Fig. 2B). The inhibitory effect of rosiglitazone (≥5 μM) on basal proliferation was confirmed in MDCK monolayer cultures using a BrdU incorporation assay (Fig. 2C).

To study changes in apoptosis, we stained cysts using an antibody to cleaved caspase-3 before (day 3) or after (day 6) the onset of lumen formation (day 4). As shown in Fig. 2D, rosiglitazone increased the percentage of cysts with apoptotic cells by over twofold at both time points. Another striking difference was a major shift in the location of apoptotic cells away from luminal (DMSO) to multiple nonluminal areas following rosiglitazone treatment (Supplementary Fig. S1; all supplemental material for this article is accessible online at the journal web site). Rosiglitazone did not increase the basal

Fig. 2. Rosiglitazone inhibited proliferation, induced apoptosis, and decreased the percentage of single central lumen cysts in MDCK II cells. A: total cell number of MDCK II cysts from triplicate wells after 13 days following treatment with rosiglitazone (0.5–100 μM) or DMSO. *P < 0.05 vs. DMSO (100%). B: rosiglitazone (20 μM for 8 days) significantly suppressed cell proliferation compared with DMSO-treated cysts, as quantified as the number of Ki-67-positive cells per cyst. C: rosiglitazone significantly inhibited cell proliferation in monolayer cultured MDCK cells. A small but significant dose-dependent effect was seen compared with monolayer cells by BrDU incorporation assays at doses ≥5 μM. Data shown represent the mean of three experiments performed in triplicate. *P < 0.05 vs. DMSO control. D: there was a significant increase in the percentage of cysts with apoptotic cells (cleaved caspase-3 positive) following rosiglitazone (20 μM) treatment in MDCK cysts before (day 3) or after (day 6) lumen formation. E: rosiglitazone (20 μM) did not significantly alter the apoptotic rate (cleaved caspase-3 positive) of monolayer MDCK cells. Data are shown from 3 experiments performed in triplicate. F: MDCK II cells formed cysts with a well-developed central lumen following plating into type I collagen gels. Incubation with rosiglitazone (0.5–100 μM for 13 days) induced MDCK cysts with multiple ectopic lumen and cellular aggregates without a discernable lumen in a dose-dependent manner. *P < 0.01 vs. DMSO-treated cells for single-lumen cysts.
apoptotic rate in MDCK monolayers (Fig. 2E). This suggests that the increase in apoptosis observed in 3D culture might be secondary to changes in cell polarity (see below).

**Rosiglitazone disrupts central lumen formation in MDCK cysts.** Besides the effects on proliferation and apoptosis, rosiglitazone-treated cells displayed a clear defect in central lumen formation. Rosiglitazone reduced the percentage of single central lumen cysts (91.8% in the DMSO group vs. 0–53.3% in the rosiglitazone groups, *P < 0.01*). There was a corresponding increase in the percentage of multiple lumen cysts (1% in the DMSO group vs. 12.8–24.4% in the rosiglitazone groups, *P < 0.01*) and cell aggregates without obvious lumen (7.23% in DMSO vs. 24.3–87.24% in rosiglitazone groups, *P < 0.01*, Fig. 2F). This effect was observed at doses below 10 μM (as low as 0.5 μM) and therefore distinguishable from the effects on cell proliferation and apoptosis.

In addition to multiple lumens, we observed short irregular tubular protrusions in a minority (~20%) of rosiglitazone-treated cysts. These were not present in control cells and varied in length from 5 to 40 μm (Supplementary Fig. S2, for example).

**Rosiglitazone induces mislocalization of apical and basolateral membrane proteins in MDCK cysts.** The protein gp135/podocalyxin is expressed at the apical surface before lumen formation is visible and marks the site of future lumen formation. In view of the defect in apical lumen formation, we followed the expression of this protein in cells from day 2 after plating. As reported previously, we observed localization of gp135 to the cell-cell borders of dividing cells in the DMSO controls (18). With rosiglitazone, however, gp135 became mislocalized to multiple intracellular vesicles and to the cellular edges of outer protrusions (Fig. 3A). By day 12, multiple gp135-positive lumen structures had formed, unlike a single central lumen in DMSO controls (Fig. 3B). At intermediate time points (day 3, day 6, day 8), we observed gp135 expression at areas of membrane protrusions rather than in the central lumen or preluminal membranes (Supplementary Fig. S2).

This alteration in gp135 localization was accompanied by the mislocalization of the basolateral protein E-cadherin in some cysts. By day 8, ~30% of rosiglitazone-treated cysts displayed apical E-cadherin staining in addition to basolateral staining (Supplementary Fig. S3).

**Rosiglitazone randomizes mitotic spindle orientation.** Misalignment of the mitotic spindle between dividing cells has been reported to result in multiple lumen cysts through the targeting of apical patches to aberrant sites (12). We studied the effects of rosiglitazone on the mitotic spindle orientation of MDCK cells in collagen gels, by measuring the spindle angles in dividing cells as shown in Fig. 4B. In control cysts, the majority of the mitotic spindles are oriented within the tissue plane (perpendicular to the centroid of the cyst) with a mean angle of 73.8 ± 3.5° (*n* = 30) (Fig. 4, A and C). Following rosiglitazone treatment, mitotic spindle angles became randomized, with a mean angle of 44.0 ± 5.1° (*n* = 27) (Fig. 4, A and C).

**Rosiglitazone retards the reorientation of centrosomes.** Initial experiments indicated that rosiglitazone did not alter the gross morphology of the microtubular network in MDCK cells (data not shown). However, we hypothesized that rosiglitazone might alter specific microtubular functions such as centrosome migration and polarity. Normally, the centrosomes are located randomly following seeding onto collagen I-coated coverslips. As apical polarity is established through cell-matrix cues, cellular organelles including centrosomes and Golgi will reorient to the apical side of the nucleus.

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**Fig. 3.** Rosiglitazone induced ectopic expression of apical marker gp135 in MDCK II cells in 3-dimensional (3D) culture. A: *z*-scans (i, ii, and iii) and 3D reconstructed images (iv) of day 2 MDCK II cells treated with DMSO or rosiglitazone showing that gp135 is normally localized between dividing cells but shows a randomized location after rosiglitazone treatment. B: central lumen apical gp135 (green) expression in DMSO-treated cysts on day 12 compared with gp135 expression on multiple ectopic lumens after rosiglitazone. F-actin (red) staining highlights lumen structures. Scale bar = 20 μm.
Rosiglitazone alters primary cilia expression and length. Primary cilia are enucleated from the mother centriole or basal body. In view of the defects in spindle orientation and centrosome reorientation, we hypothesized that rosiglitazone might result in abnormalities in cilia structure. Preliminary experiments indicated that primary cilia in MDCK cysts were only visible from day 10 to day 11 in 3D culture (data not shown). Cilia length was similar at day 12 in control and treated cysts. However, treated cysts expressed significantly shorter cilia at days 14 and 17 (Supplementary Fig. S4). In monolayer culture, MDCK cells expressed longer cilia compared with 3D culture and at an earlier time point (Supplementary Fig. S4, B and D). Rosiglitazone similarly reduced cilia length in MDCK monolayers (DMSO: 12.45 ± 0.36 μm vs. rosiglitazone: 7.49 ± 0.17 μm, Supplementary Fig. S4D) and increased the percentage of cells without detectable primary cilia by 20% (DMSO: 8% vs. rosiglitazone: 28%, Supplementary Fig. S4E).

Rosiglitazone inhibits activation of the small GTPase Cdc42 and alters its subcellular localization in MDCK cysts. The small GTPase Cdc42 has been recently shown to play a critical role in the establishment and maintenance of apical polarity and lumen formation in epithelial cells. In particular, knockdown of Cdc42 induced a multiple lumen phenotype in MDCK and Caco-2 cells (12, 18). We next investigated whether alterations in Cdc42 localization, expression, or activation might underlie the disruption of apical lumen formation induced by rosiglitazone.

As shown in Fig. 6A, Cdc42 in control cells localized in an almost identical pattern to gp135, i.e., between dividing cells before (day 3), and lining the luminal surface after, lumen formation (day 6) as previously reported (18). Similar to its effect on gp135 (Fig. 3), rosiglitazone randomized Cdc42 localization initially to protrusions at the edges (day 3) and to multiple lumen surfaces (day 6). Although total Cdc42 expression was unchanged, Cdc42 activation (GTP-bound) in treated cysts was profoundly inhibited by rosiglitazone (Fig. 6B). By contrast, rosiglitazone had no effect on Cdc42 activation in MDCK monolayer cells (Supplementary Fig. S5).

Inducible expression of Cdc42 mutants interferes with apical expression of gp135 and central lumen formation. In a previous study, it was reported that MDCK cells inducibly express-
ing (Tet-Off) either dominant-negative (Cdc42N17) or constitutively active Cdc42 (Cdc42V12) (Fig. 7A) had a reversal of apicobasal polarity in 3D culture, as reflected by phalloidin staining (27). However, gp135 localization or the nature of lumen formation was not reported. Using the same clones, we found gp135 staining at the basolateral surface following Cdc42 induction (Fig. 7B). There was also a significant reduction in the number of single central lumen cysts compared with controls (85.5% in control cells, 1.33% in Cdc42N17 cells, and 4.667% in Cdc42V12 cells, \( P < 0.001 \)). Loss of luminal gp135 expression was more profoundly seen in Cdc42N17 cells (73.8% in Cdc42N17 cells vs. 35.3% in Cdc42V12 cells, \( P < 0.01 \)). These results suggest that basal Cdc42 activity is essential for apical gp135 expression and central lumen formation (Fig. 7B).

Rosiglitazone induces a multilumen phenotype in Cdc42-expressing cells. Similar to MDCK II cells, MDCK T23 cells displayed a clear defect in central lumen formation following rosiglitazone exposure (20 \( \mu \)M for 7 days) (Fig. 7C). The percentage of single lumen cysts (85.5% DMSO vs. 7.7% rosiglitazone, \( P < 0.01 \)) was reduced with a corresponding increase in the percentage of multilumen cysts and cell aggregates without obvious lumen. Rosiglitazone treatment had little effect on lumen formation in Cdc42V12 cells. However, in Cdc42N19 cells, it increased the proportion of multiple lumen cysts (25% untreated vs. 62% treated) compared with a higher percentage of aggregates present in untreated cells (Fig. 7C). This was associated with relocalization of gp135 from the basolateral surface to the internal lumen (Fig. 7B).

**DISCUSSION**

The genesis of epithelial polarity is critical for the formation of tubular structures during development. Two major events seem to be important for epithelial morphogenesis. Initial cell-ECM interaction orientates the apicobasal cell axis. This is followed by the formation of an apical lumen through the genesis, polarized delivery, and exocytosis of apically destined vacuoles or vesicles. Formation of the central lumen can occur by either cavitation (necessitating luminal apoptosis) or hollowing (exocytosis of preformed apical vesicles) depending on the model used (19).

In this study, we found that rosiglitazone inhibited MDCK cyst growth by inhibiting proliferation and, unexpectedly, by interfering with central lumen formation. Incubation with rosiglitazone resulted in misorientation of the mitotic spindle in dividing cells and a mistargeting of gp135, an apical marker protein at a very early stage of cyst formation. These two effects resulted in the formation of multiple lumens instead of a single central lumen.

The Rho GTPase Cdc42 is considered a master regulator of cell polarity (11). In fibroblasts, Cdc42 acts upstream of Rac and Rho to control the formation of actin-based structures and thus cell motility (22). Cdc42 has also been shown to regulate the constitutive exit of proteins from the trans-Golgi network (20). Recently, several new lines of evidence indicate that Cdc42 also plays an essential role in the establishment of the apical surface. First, it regulates the spatial exocytosis of apical proteins via phosphatidylinositol 4,5-bisphosphate-dependent annexin 2 targeting (18). Sec-
ond, it controls spindle orientation during cell division in 3D culture (12). In vivo, Cdc42 has been shown to be essential for pancreatic tubulogenesis and indirectly specifies cell fate in pancreatic development (14).

Our results clearly indicate that Cdc42 activation is inhibited and its normal apical localization in 3D culture randomized by rosiglitazone treatment. This is likely to result in loss of apical targeting of gp135 as well as changes in oriented cell division. In previous studies, knockdown of Cdc42 by small interfering RNA delayed fusion of gp135-containing vesicles with the plasma membrane (18). Cdc42 is also known to be required for the exocytosis of secretory vesicles in neuroendocrine cells (16) and yeast (1). These data place Cdc42 as an upstream regulator for gp135 targeting.

Recently, a role of Cdc42 in regulating the fidelity of mitotic spindle alignment and asymmetric abscission between dividing epithelial cells has been shown (12). Oriented cell division in this model was critical for the maintenance of a central lumen since misalignment of the mitotic spindles resulted in a multilumen phenotype (as observed in our study) through the targeting of apical patches to aberrant sites (12). E-cadherin-mediated cell contacts are known to activate Cdc42 and to regulate spindle orientation in MDCK cells in an APC-dependent but Par3/aPKC-independent manner (10, 15). It seems likely that multiple mechanisms could be involved in correct spindle positioning in epithelial cells.

Both of these functions of Cdc42 rely on the correct spatiotemporal localization of Cdc42 (3, 7, 18, 26). Indeed, we observed that Cdc42 localized to regions of membrane protrusions following rosiglitazone treatment (Fig. 6), a feature not observed in control cells. Although these tubular protrusions did not elongate continuously, they may represent the response to ectopic Cdc42 localization such as that described at the leading edge of migrating cells (35) or polarized growth (11). Of relevance, these early structures were transient and multiple ectopic lumen were visible later. This suggests that the ectopic expression of Cdc42 itself is insufficient to stimulate continuous cell migration but that the intracellular ectopic localization of Cdc42 was sufficient to stimulate lumen formation. The latter may explain both the overall increase in apoptotic rate and the ectopic “extraluminal” nature of apoptotic cells seen (Fig. 2D, Supplementary Fig. S1).

Cdc42 also regulates centrosome reorientation in migrating fibroblasts by both actin and microtubular pathways (4, 29). These could explain the defects in centrosomal migration and shorter cilia length we observed in longer cultures. Defects in centrosome migration and ciliary structure or function have been linked to human diseases associated with a cystic phenotype. We suggest that correct centrosome orientation and polarity are linked to proper cilia development and apical lumen formation. Potentially, changes in
the rate of centrosomal migration could have contributed to loss of oriented cell division in our model.

To confirm the role of Cdc42, we examined MDCK cells expressing either dominant-negative (Cdc42N19) or constitutively active (Cdc42V12) Cdc42. Similar to rosiglitazone-treated control cells, both mutants showed mislocalization of gp135 and loss of a single-lumen phenotype, supporting a critical role for Cdc42 in both functions. However, rosiglitazone did increase the percentage of cells with a multilumen phenotype. Rosiglitazone had little effect in Cdc42V12 cells but increased the proportion of Cdc42N17 cells with a multilumen phenotype. C: quantification of cyst lumen phenotype in all 3 lines following DMSO or rosiglitazone treatment for 6 days. Individual cysts were classified as either single lumen, multiple lumen, or aggregates (no lumen) by phase-contrast microscopy and expressed as a percentage of the total numbers of cysts counted (n = 25). Cdc42N17 cells formed a higher percentage of aggregates than Cdc42V12 and a lower percentage of single-lumen cysts. Following rosiglitazone treatment, there was a higher percentage of Cdc42N17 cells with a predominant multiple-lumen phenotype but no significant change in Cdc42V12 cells.

Fig. 7. Inducible expression of mutant Cdc42 leads to mislocalization of gp135 and loss of a central-lumen phenotype. A: control (T23) and Cdc42 mutant MDCK cells showing the absence of Cdc42-myc in the presence of tetracycline (+) but its induction following tetracycline withdrawal (-). Lysates were blotted with an anti-myc (top) and anti-Na⁺/K⁺-ATPase (bottom) antibody as a loading control. B: control (T23) and Cdc42 mutant MDCK cells in 3D culture at 7 days following DMSO or rosiglitazone (20 μM) treatment for 6 days. Immunofluorescence for gp135 (green) and phase-contrast microscopy (lumen) is shown in adjacent panels for the same cysts. Both Cdc42V12 and Cdc42N17 mutants show mislocalization of gp135 to the basolateral surface and a multilumen phenotype. Rosiglitazone had little effect in Cdc42V12 cells but increased the proportion of Cdc42N17 cells with a multilumen phenotype. C: quantification of cyst lumen phenotype in all 3 lines following DMSO or rosiglitazone treatment for 6 days. Individual cysts were classified as either single lumen, multiple lumen, or aggregates (no lumen) by phase-contrast microscopy and expressed as a percentage of the total numbers of cysts counted (n = 25). Cdc42N17 cells formed a higher percentage of aggregates than Cdc42V12 and a lower percentage of single-lumen cysts. Following rosiglitazone treatment, there was a higher percentage of Cdc42N17 cells with a predominant multiple-lumen phenotype but no significant change in Cdc42V12 cells.

In summary, we report two potential mechanisms by which PPAR agonists can retard cyst formation in MDCK cells, i.e., by disrupting oriented cell division and lumen formation. These changes are likely to underlie the inhibitory effects of these compounds on cyst formation in vivo.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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