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Glycosphingolipid synthesis is essential for MDCK cell differentiation

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ABSTRACT

Glycosphingolipids (GSLs), which are highly concentrated at the apical membrane of polarized epithelial cells, are key components of cell membranes and are involved in a large number of processes. Here, we investigated the ability of hypertonicity (high salt medium) to induce Madin–Darby Canine Kidney (MDCK) cell differentiation and found an increase in GSL synthesis under hypertonic conditions. Then, we investigated the role of GSLs in MDCK cell differentiation induced by hypertonicity by using two approaches. First, cultured cells were depleted of GSLs by exposure to D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP). Second, cells were transfected with an siRNA specific to glucosylceramide synthase, the key enzyme in GSL synthesis. Exposure of cells to both treatments resulted in the impairment of the development of the apical membrane domain and the formation of the primary cilium. Enzymatic inhibitions of the *de novo* and the salvage pathway of GSL synthesis were used to determine the source of ceramide responsible of the GSL increase involved in the development of the apical membrane domain induced by hypertonicity. The results from this study show that extracellular hypertonicity induces the development of a differentiated apical membrane in MDCK cells by performing a sphingolipid metabolic program that includes the formation of a specific pool of GSLs. The results suggest as precursor a specific pool of ceramides formed by activation of a Fumonisin B1-resistant ceramide synthase as a component of the salvage pathway.

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1. Introduction

Sphingolipids (SLs) are typically found in eukaryotic cells, where they comprise a small but vital fraction of membrane lipids. Glycosphingolipids (GSLs), a class of SLs, are believed to play important roles in a variety of cellular processes, such as cell recognition, development and differentiation [1].

SLs contain a ceramide moiety as their core; the addition of a phosphocholine head group generates sphingomyelin, while the addition of sugars to the ceramide moiety generates GSLs. When the head group contains the negatively charged sugar sialic acid, the lipids are referred to as gangliosides, whereas when they lack sialic acid, they are called neutral GSLs. Although GSLs commonly contribute less than 5% to the total cellular lipid pool, they are highly enriched in the outer leaflet of the apical plasma membrane domain of polarized epithelial cells. The high apical content of GSLs is thought to stabilize the membrane. They are uniquely suited for this stabilizing function. Besides their capacity to form hydrogen bonds between the amide, the carboxyl and the hydroxyl groups of the sphingosine, the hydration of oligosaccharide hydrophilic head group can favor the formation of intermolecular hydrogen bonds, thus providing another level of molecular stabilization [2–4]. Because GSLs have a tendency to self-segregate themselves as "raft", they act as primary sorters of apical proteins at the Golgi complex by promoting their incorporation into apically directed carrier vesicles [5,6].

Besides being a functionally and structurally specialized membrane domain, the apical surface of epithelial cells serves to delineate the tubular lumen in renal collecting duct cells. A crucial event for lumen formation is the accumulation of anti-adhesive factors in the apical surface to prevent the membrane sticking of adjacent cells. Because of its highly negatively charged ectodomain [7,8], podocalyxin, also known as gp135, has been confirmed to assess this function [9], and thus to initiate the process of morphological differentiation of polarized epithelial cells. The final stage in the differentiation process is the outgrowth of a primary cilium from the apical surface of the cells

Abbreviations: MDCK, Madin–Darby Canine Kidney; GSLs, glycosphingolipids; SLs, sphingolipids; FBS, fetal bovine serum; DMEM/F12, Modified Eagle's Medium/F-12; siRNA, small interfering RNA; SCR, scrambled; TLC, thin-layer chromatography; GlcCer, glucosylceramide; LacCer, lactosylceramide; TrihexCer, trihexosylceramide; GCS, glucosylceramide synthase; D-PDMP or L-PDMP, D- or L-*threo*-1 phenyl-2-decanoylamino3-morpholino-1 propanol; SPT, serine palmitoyl transferase; CerS, ceramide synthase; CS, L-cycloserine; FB1, Fumonisin B1; CERT, ceramide transport protein

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[10]. The cilium represents a distinct sub-domain of the apical surface that sequesters a unique complement of proteins and lipids [11].

Madin–Darby Canine Kidney (MDCK) is a cell line derived from dog renal collecting ducts [12] that has retained the potential to achieve differentiation under certain conditions [13,14]. External hypertonicity is the physiological condition for renal collecting duct cells and it has been demonstrated that, under hypertonicity, MDCK cells express a myriad of functional proteins such as channels, pumps and co-transporters, typical of functionally differentiated cells [15–20].

To be fully functional polarized, cells require that proteins reside in their specific membrane domain (apical or basolateral); we thus hypothesize that concomitantly with the induction of functional proteins, hypertonicity must induce changes in the lipid membrane environment where functional proteins could be recruited.

Considering the enrichment of GSLs in the apical membrane of polarized cells, and the above-mentioned involvement of electrolyte environment in the expression of the proteins that carry out the function of polarized cells, we investigated the involvement of GSL synthesis in the differentiation of MDCK cells. In this study, we provide evidence that external hypertonicity induces the development of the differentiated phenotype of MDCK cells by inducing a program of sphingolipid metabolism that includes increased expression and activity of glucosylceramide synthase (GCS) acting on a specific pool of ceramides. This is the first description of the direct involvement of GSL synthesis in the physiological induction of collecting duct cell differentiation.

2. Materials and methods

2.1. Materials

D-[1-^{14}C]Galactose (specific activity 50–60 mCi/mmol) and [1-^{14}C] stearic acid (specific activity 55 mCi/mmol) were from American Radiolabeled Chemicals, D-erythro [3-^{3}H]sphingosine (specific activity 21.2 Ci/mmol) and [^{14}C]palmitic Acid (specific activity 55 mCi/mmol) were from Perkin Elmer Life Sciences. TLC silica gel plates were from Merk. Dulbelcco's Modified Eagle's Medium/F-12 (DMEM/F12), penicillin-streptomycin, Lipofectamine 2000, GCS siRNA, scrambled siRNA and NBD C6-ceramide were purchased from Invitrogen. Fetal bovine serum (FBS) was from Natocor. Fumonisin B1 and L-Cycloserine were from Sigma. L-PDMP and D-PDMP were from Matreya. Monoclonal mouse anti-gp135 was kindly donated by Dr. Ojakian (SUNY Downstate Medical Center, USA). Rabbit anti-α-catenin and mouse anti-acetylated tubulin were from Sigma, rabbit anti-Zo-1 from Zymed, mouse anti-Na/K ATPase from Santa Cruz Biotechnology, and mouse anti-actin from Chemicon. Secondary antibodies anti-mouse Alexa 546 and anti-rabbit Alexa 488 were from Invitrogen. Vectashield Mounting Medium was from Vector Laboratory. The polyclonal rabbit antiserum against GCS was a generous gift from Dr. Marks (Thoracic Diseases Research Unit, Rochester, USA). Peroxidase-labeled anti-rabbit antibody and ECL plus were from GE Healthcare. RT-PCR reagents were purchased from Promega. All reagents were of analytical grade or higher.

2.2. Cell culture

MDCK cells (2×10^5/35 mm dish) were grown in DMEM/F-12 supplemented with 10% FBS, penicillin (100 μg/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified 5% CO_2 atmosphere. After 24 h, the medium was replaced by DMEM/F12 containing 0.5% FBS and incubated for another 24 h to reach confluence. Then, cells were switched to a hypertonic medium (300 mM NaCl) and further incubated for 24, 48 or 72 h in the presence or absence of the various inhibitors of sphingolipid synthesis. L-Cycloserine, Fumonisin B1 and D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) were used to inhibit serine palmitoyl transferase (SPT), ceramide/DH ceramide synthase (CerS), and glucosylceramide synthase (GCS) respectively. L-PDMP was used as the negative control of the GCS inhibition exerted by D-PDMP. After treatments, cells were trypsinized, counted, and cell viability controlled with the trypan blue exclusion procedure.

2.3. Transfection with small interference RNA (siRNA)

MDCK cells (1.5×10^5/35 mm dish) were grown in DMEM/F-12 containing 10% FBS without antibiotics. After 24 h, cells were transfected with 400 nM double-stranded siRNAs for GCS or scrambled sequence (SCR) using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h, transfection reagents were washed out and the medium was replaced by DMEM/F12 containing 0.5% FBS and incubated for another 24 h to reach confluence. Then, the medium was replaced by DMEM/F12 containing 1% FBS and incubated in the presence or absence of 300 mM NaCl for 24, 48 or 72 h. The sequences of siRNAs for GCS were CCAGGAUAUGAAGUUGCAA (sense) and UUGCAACUUCAUAUCCUGG (antisense) and the sequences for SCR were CAGUCGCGUUUGCGACUGG (sense) and CCAGUCGCAAACGCGACUG (antisense).

2.4. Labeling and isolation of glycosphingolipids

GSLs were labeled with feeding cells with D-[1-^{14}C]galactose (2 μCi/ml) for 24 h or [^{14}C]palmitic acid (25 nCi/ml, 1 h or 24 h) before trypsinization and extracted by the method of Bligh and Dyer. Glycerophospholipids were removed by saponification in alkaline methanol followed by reextraction and evaporation. Aliquots of sphingolipids were spotted onto a thin-layer chromatography (TLC) plate, developed in chloroform/methanol/0.22% $CaCl_2$ (60:35:8; vol/vol/vol) and visualized by autoradiography. The corresponding radioactive spots were scraped off the TLC plates for additional measurement by liquid scintillation counting. Data were normalized to the number of counts incorporated every 10^6 cells.

The mass levels of GSLs were determined by a colorimetric assay. TLC plates were dipped into a 3% cupric acetate (w/v)-8% phosphoric acid (v/v) solution for 10 s, air-dried and heated at 150 °C for 5 min, scanned and analyzed by densitometry [21].

2.5. Glucosylceramide synthase assay

Cells were incubated with 5 μM NBD C6-ceramide for 2 h, and total lipids were extracted according to the method of Bligh and Dyer. The chloroform phase was collected and lipids resolved by TLC in chloroform/methanol/water (65:25:4, vol/vol/vol). NBDC6-GlcCeramide fluorescence was measured using a PerkinElmer LS55 fluorescence spectrometer at $\lambda_{excitation}$ 466 nm and $\lambda_{emission}$ 536 nm. The amounts of GlcCer were quantitated with a standard curve and the results are expressed as pmol GlcCer/mg protein [22].

2.6. (Dihydro) ceramide synthase assay

The relative activity of DH-Cer/Cer synthases was determined as described by Wang et al. [23] with slight modifications. Cultured cells were incubated in the presence of 1 μCi of [^{3}H]sphingosine. After 1 h of incubation, the cells were scraped from the well and the lipids were extracted as described above. The [^{3}H]ceramide was separated from the substrate by TLC developed with n-butanol/acetic acid/water (60:20:20, v/v/v) and visualized by autoradiography, and the amount of radioactivity was determined by scintillation counting. Data were normalized to the amount of [^{3}H]ceramide formed per mg of protein.

For a partial characterization of the enzyme, cells were incubated in the presence of [^{14}C]stearic acid or [^{14}C]palmitic acid (25 nCi/ml,

24 h) before trypsinization and extracted by the method of Bligh and Dyer. After saponification and reextraction [^{14}C]ceramide formed was separated by TLC and quantified as described above and data were normalized to the number of counts incorporated every 10^6 cells.

2.7. Confocal immunofluorescence microscopy

MDCK cells grown on coverslips were fixed in 4% paraformaldehyde for 20 min (or methanol/acetone for Na/K ATPase), and permeabilized with 0.1% (v/v) Triton X-100 for 20 min. After blocking, cells were incubated for 90 min with 1:10 of mouse anti-gp135, 1:2000 of rabbit anti-α-catenin, 1:500 of rabbit anti-Zo1, 1:500 mouse anti-acetylated tubulin or 1:200 mouse anti Na/K ATPase at room temperature. Primary interactions were detected by using anti-rabbit Alexa 488 and anti- mouse Alexa 546-conjugated antibodies. After 60 min, the coverslips were mounted onto microscope glass slides with Vectashield Mounting Medium and examined with an Olympus FV300 confocal microscope (model BX61) and processed using the FluoView version 3.3 software. Optical sections obtained with an oil immersion 60×1.4 NA objective were 0.5 μm. For xz assembled images and 3D reconstruction, Image J software was used. Fluorescent quantifications (Fig. 2) were performed with Image-Pro plus version 4.5 software.

2.8. Western blotting

Cells were collected by trypsin-EDTA treatment and then resuspended in lysis-buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM VO_4, 10 μg/ml aprotinin). Lysis was completed by passing cells through a 27-gauge needle and sonication. Protein samples of 50 μg were incubated with 4× Laemmli buffer at 100 °C for 5 min, and resolved in a 12% SDS-polyacrylamide gel. Proteins were electrophoretically transferred to PVDF membranes, blocked with 5% non-fat milk in TBS-Tween and incubated at 4 °C with rabbit antiserum against GCS (1/1000) for 18 h or anti-actin (1/2500) for 1 h. The blots were washed with TBS-T and incubated with secondary antibody conjugated with horseradish peroxidase. Detection was performed using ECL plus reagent.

2.9. Reverse transcription-PCR

Total RNA was extracted from control and transfected cells using the SV total RNA isolation system in accordance with the manufacturer's instructions. Equal amounts of RNA (500 ng) were used to synthesize the first-strand cDNA using the reverse transcriptase system and then PCR. Primers used for PCR were 5′ TCATGATGATCCAGCCATTG 3′ (forward) and 5′ TAAGCCCTCCTGCTTGATCT 3′ (reverse) for GCS and 5′ CAAAGCCAACCGTGAGAAG 3′ (forward) and 5′ CAGAGTCCATGACAATACCAG 3′ (reverse) for β-actin. RT-PCR products were resolved on 2% agarose-gels.

3. Results

3.1. Hypertonicity induces an increase in glycosphingolipid synthesis in MDCK cells

To investigate GSL metabolism, we analyzed the incorporation of [^{14}C]galactose in confluent MDCK cells either subjected to isotonic medium (150 mM NaCl) or exposed to high salt medium (300 mM NaCl) for 24 h or 48 h. The endogenous GSLs were also determined. MDCK cells presented a basal level of GSL synthesis with most of the radioactivity accounted for by the neutral GSLs (glucosylceramide (GlcCer), lactosylceramide (LacCer) and trihexosylceramide (TrihexCer)), and 25% of the radioactivity accounted for by the acidic GSL GM3. The endogenous level of each GSL appeared almost equally distributed among the neutral GSLs and the acidic GM3 (Fig.1A). The exposure of confluent MDCK cells to hypertonicity resulted in an increase in the total [^{14}C]GSLs and in the endogenous mass at 24 h, which persisted after 48 h of treatment (Fig. 1B), with no qualitative changes in its profile. When individual GSLs were analyzed, we observed that, under hypertonicity, [^{14}C]GlcCer and its endogenous mass peaked at 24 h, while the synthesis of the other GSLs remained similar to those of the control conditions. After 48 h of treatment, the level of [^{14}C]GlcCer returned to control values, whereas [^{14}C]LacCer and [^{14}C]TrihexCer and their respective endogenous mass were increased. No significant changes were observed in GM3, whose level remained constant in all the experimental conditions (Fig. 1C).

These results demonstrate that external hypertonicity induces an increase in GSL synthesis initiated with a rise in GlcCer to be further selectively channeled to form more complex neutral GSLs.

3.2. Glucosylceramide synthase is regulated by hypertonicity

GlcCer, a central back-bone for most complex GSLs, is synthesized by GCS, the rate-limiting enzyme in the biosynthetic pathway. GCS is an integral protein initially considered resident of cis/medial-Golgi membrane [24], but it has been recently reported to be more widely distributed in the Golgi apparatus [25]. The sequence analyses suggest that GCS is a Type III protein but not structurally similar to other glycosyltransferases [26]. To investigate whether the hypertonicity-induced increase in GSL synthesis occurred by regulation of GCS at the transcriptional level, we next examined GCS mRNA levels. RT-PCR analysis showed a basal level of gene expression which significantly increased after 24 h of hypertonicity (Fig. 1D). Thereafter, a Western blot analysis was performed to determine whether the increase in GCS mRNA level was reflected by an enhanced protein level. Indeed, a more than two-fold increase in protein concentration was observed after the high salt-treatment (Fig. 1E).

Taken together, these results demonstrate that hypertonicity induces an increase in GCS gene expression, which is reflected by an increase in GCS mRNA and protein content.

3.3. Hypertonicity induces MDCK cell differentiation

It has been previously reported that extracellular hypertonicity induces the expression of proteins typical of differentiated renal tubular collecting duct cells [15,20]. However, no evidences have been reported about the acquisition of a morphologically differentiated phenotype which is reflected by apical membrane development and primary cilium formation. To investigate whether the hypertonic medium induces the morphological differentiation of MDCK cells, the cells were immunolabeled with anti-gp135 and anti-α-catenin as apical and basolateral markers respectively, and visualized by confocal microscopy. Serial sections of 0.5 μm were collected from the bottom to the top of the cell monolayers. We considered as the most basal and most apical plane the first and the last α-catenin-positive sections, respectively. Planes were numbered as 1 for the most basal and 14 or 18 for the most apical, for isotonicity- or hypertonicity-cultured monolayers, respectively. The fact that in control cells the total number of positive planes for the α-catenin signal was 14 and that in the cells subjected to hypertonicity the number increased to 18 indicates that under hypertonicity the cells acquired the classical columnar shape typical of the tubular epithelium [27]. Representative images from basal (plane 2), middle (plane 5) and apical positions (plane 11 in control monolayers and plane 14 in hypertonicity-subjected monolayers) of optical sections are presented in Fig. 2A. The confocal images show that both under isotonicity and after 48 h of hypertonicity, α-catenin appeared outlining the cells from the most basal to the most apical confocal planes, thus reflecting lateral membrane localization. Some α-catenin positive signal was also found intracellularly distributed under isotonicity but not under

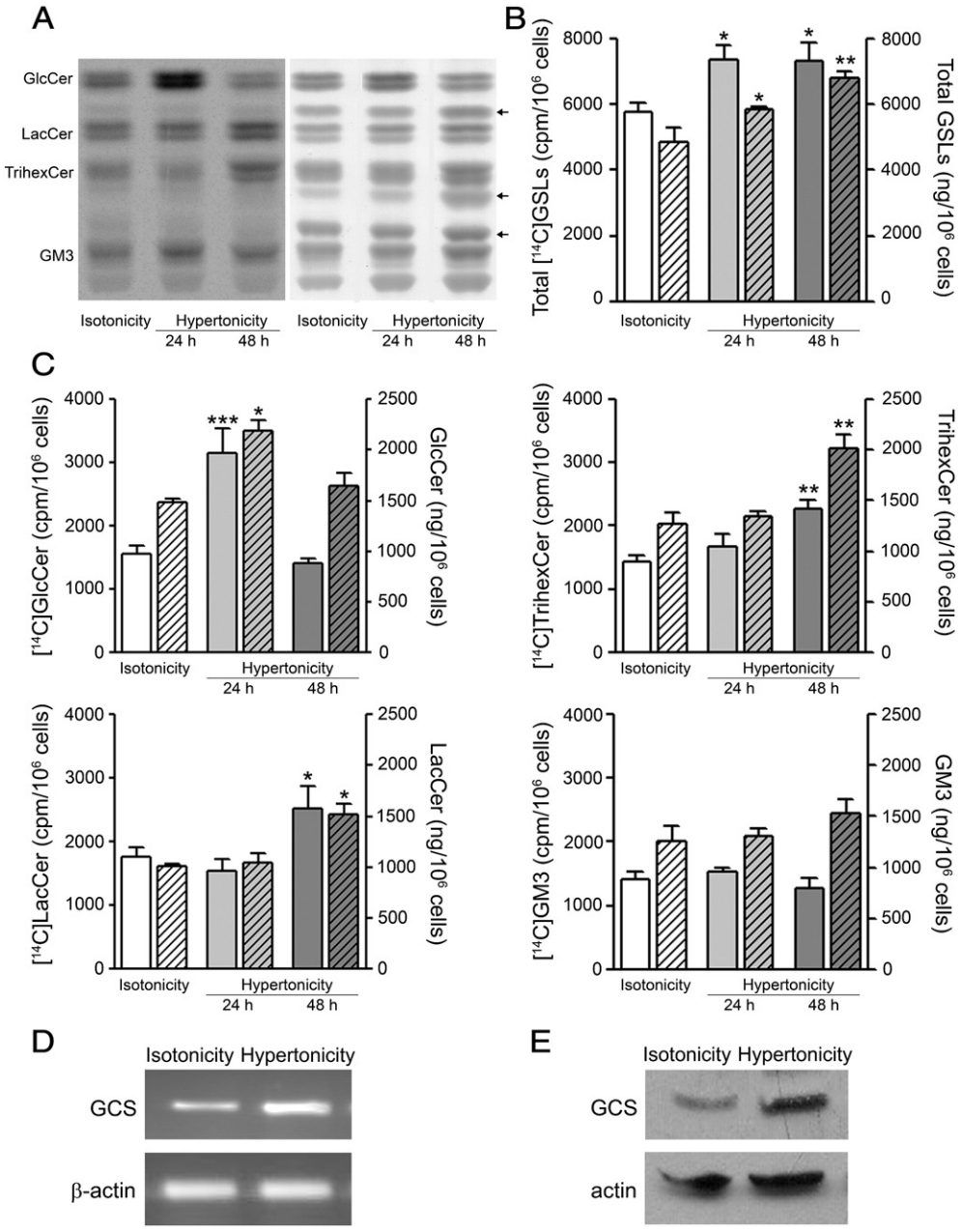

Fig. 1. Metabolism of glycosphingolipids (GSLs) by MDCK cells under hypertonic conditions. A. GSL analysis from MDCK cells. Confluent MDCK cells were radiolabeled for 24 h with [^{14}C]galactose under isotonicity (150 mM NaCl) or hypertonicity (300 mM NaCl) for 24 or 48 h. The lipid extracts belong to an equal number of cells. A lipid autoradiography (left) and the chromatogram visualized with 3% cupric acetate — 8% phosphoric acid reagent (right) of a representative TLC are shown. Arrows indicate non-GSLs. B. Quantitative analysis of total [^{14}C]GSLs and mass levels from MDCK cells. Endogenous GSLs were detected with 3% cupric acetate — 8% phosphoric acid reagent and the results are represented by dashed bars referenced to the right axis of the graph. The data represent mean ± SEM of three to five values (*, $p<0.05$; **, $p<0.01$ *vs* isotonicity). C. Quantitative analysis of radioactive and endogenous GlcCer, LacCer, TrihexCer and GM3 under isotonicity, 24 h-hypertonicity and 48 h-hypertonicity. The data represent mean ± SEM of three to five values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; *vs* isotonicity). D. Total mRNA of MDCK cells cultured under isotonicity or 24 h-hypertonicity was extracted and GCS mRNA and β-actin mRNA levels were analyzed by reverse transcription PCR. E. MDCK cells cultured as in D were lysed and subjected to SDS-PAGE and immunoblotting with anti-GCS and anti-actin antibodies.

hypertonicity. In the same set of cells we performed the immunofluorescence analysis of gp135. Unexpectedly, under isotonicity, positive signal for gp135 appeared from the most basal planes, in some cells spread over the cell area and in others outlining the cell limits. In the middle plane, gp135 perfectly delineated the cells, acquiring an unusual distribution. The signal for gp135 appeared spread over the apical surface in most of the cells. The apical image also shows gp135-positive signal delineating the periphery of some cells (arrowhead). The overlay images show no colocalization of gp135 and α-catenin in any of the planes analyzed. In response to 48 h exposure to hypertonic medium, the immunolabeling for α-catenin appeared more clearly delineating the cell periphery all over the confocal planes examined. The immunofluorescent signal for gp135 showed its complete dissipation from the lateral domain. In contrast, gp135 appeared only accumulated at the apical membrane and with a central point devoid of gp135, typical of differentiated collecting duct epithelial cells (arrowhead). This observation is more evident in the overlay images, where the only one which outlined the cell limits was α-catenin. Confocal xz section images in Fig. 2B show these evidences more clearly. In isotonicity-cultured monolayers, the apical and lateral surfaces were not distinguishable enough, while in monolayers subjected to hypertonicity the membrane markers appeared confined to their corresponding domains, gp135 to the apical domain and α-catenin to the lateral domain (arrowheads). Moreover, in

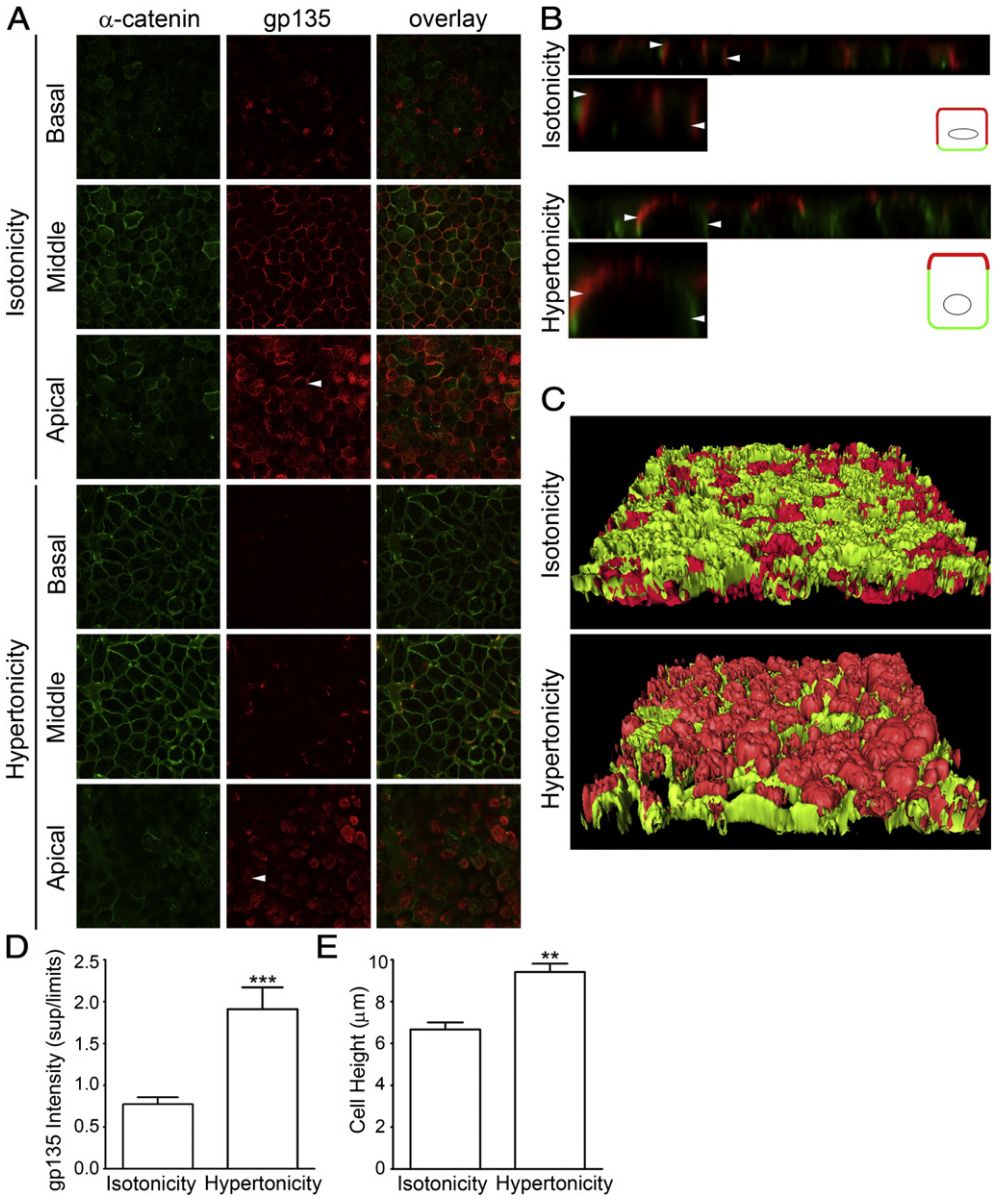

Fig. 2. Hypertonicity induces MDCK cell differentiation. A. Confluent monolayers of MDCK cells incubated under isotonic or hypertonic conditions were stained with gp135 and α-catenin antibodies, and imaged by confocal microscopy. Representative optical sections from the bottom (Basal), middle and top (Apical) of the monolayers are shown. B. Representative confocal xz section images. Pointed cells are amplified and arrowheads outline the different localization of gp135 in the monolayers subjected to isotonicity or hypertonicity. Cartoons schematically show the localization of gp135 (red) in relation to α-catenin (green). C. Representative dorsal views of 3D-rendered confocal images of MDCK cell monolayers stained for gp135 and α-catenin. D. gp135 apical and cell limit membrane fluorescence intensities were measured from confocal z sections of monolayers subjected to isotonicity or hypertonicity. Bar graphs show ratios (mean values ± SEM) of apical membrane to cell limit membrane fluorescence from three independent experiments (***, $p<0.001$). E. Cell heights were measured from cross sections (xz) of confocal images and their values (mean values ± SEM) are shown for cells subjected to isotonicity ($n=20$) and hypertonicity ($n=20$) (**, $p<0.01$).

accordance with the total number of confocal sections in the xy scan, the height of the cells subjected to hypertonicity was higher than those of control cells (Fig. 2E).

A comparative analysis of the 3D assembled confocal sections of cells allowed us to appreciate that, while under isotonicity, most cells were devoid of apical gp135 accumulation, whereas most cells subjected to hypertonicity presented an apical dome, characteristic of a differentiated columnar epithelium (Fig. 2C). Quantification of the gp135 levels measured on the cell surface in relation to the fluorescence intensity in the cell limits showed that the hypertonicity-subjected monolayers exhibited 2.5-fold more gp135 fluorescence on the cell surface than control cultures (Fig. 2D).

Taken together, these data indicate that hypertonicity induces the development of the apical membrane domain, typical of the morphologically differentiated phenotype of MDCK cells.

3.4. Glucosylceramide synthase is essential for the development of the differentiated apical membrane in MDCK cells

Taking into consideration that hypertonicity induced the development of the differentiated apical domain in MDCK cells and also triggered an increase in GCS expression and activity, we further investigated the role of GCS in the development of the hypertonicity-induced MDCK cell differentiation. To this end, we used two strategies: the pharmacological inhibition of the enzyme activity and the knockdown of its expression by transfection with small interfering RNA (siRNA). For the former, the cells were concomitantly subjected to hypertonicity and treated with D-PDMP, whereas for the latter, cells were first transfected with a specific siRNA and then subjected to hypertonicity. As negative control for D-PDMP we used L-PDMP, the inactive stereoisomer of D-PDMP

[28]. Enzyme inhibition was evaluated by measuring the enzyme activity with NDB-C6ceramide as a substrate and its knockdown was confirmed by RT-PCR. RT-PCR analysis in Fig. 3A shows that the siRNA for GCS caused a decrease in GCS gene expression compared to control cells that were transfected with a scrambled siRNA. The decrease in GCS level was confirmed by western blotting (Fig. 3B). When the enzyme activity was measured, we found that the treatment of MDCK cells with D-PDMP induced a 75% reduction in GCS activity compared to controls (L-PDMP treated cells) whereas a significant decrease was also observed in the GCS-knock-down cells (Fig. 3C). The decrease in enzyme activity was further demonstrated by analyzing the incorporation of [^{14}C]galactose to GSLs and GSL content in L- or D- PDMP-treated and GCS-knockdown cells. The pharmacological inhibition of the enzyme activity produced a 75% decrease in [^{14}C]GlcCer and [^{14}C]LacCer accompanied by a decrease in their endogenous mass. Lesser reduction was observed in TrihexCer and GM3 levels. Respect to the silencing of the enzyme, the results showed a decrease in GlcCer synthesis but not in the more complex GSLs (Fig. 3D).

To study the involvement of GSL synthesis inhibition in hypertonicity-induced MDCK cell differentiation, we determined the establishment of apicobasal polarity in both siRNA GCS and D-PDMP-treated cells subjected to 48 h hypertonicity by using confocal immunofluorescence for the apical and lateral marker

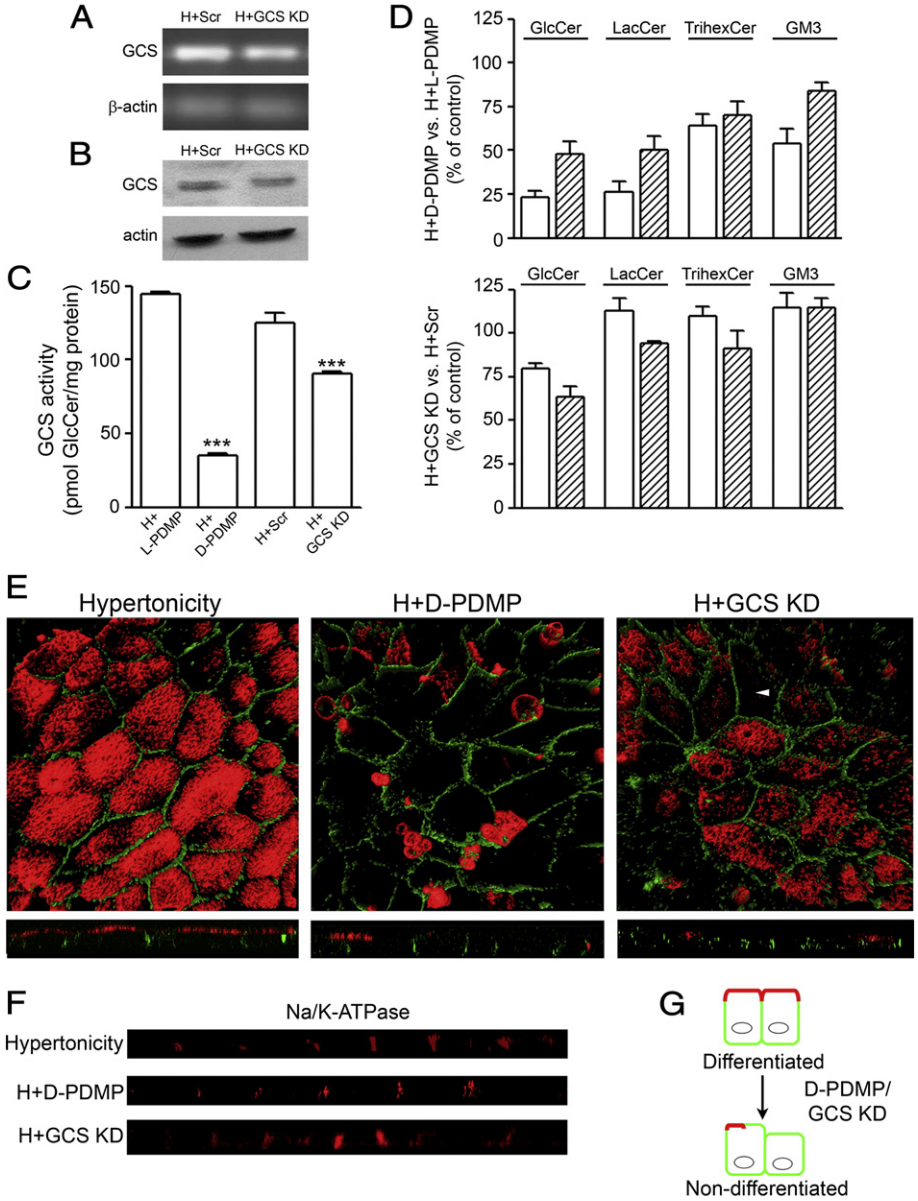

Fig. 3. MDCK cell differentiation requires glucosylceramide synthase activity. Cells were subjected to hypertonicity (H), then incubated with 5 μM D-PDMP (or L-PDMP as control) or transfected with 400 nM GCS siRNA for 24 h and then subjected to hypertonicity. A. Total mRNA of MDCK cells transfected with 400 nM siRNA of scrambled (H + Scr) or GCS subjected to hypertonicity (H + GCS KD) was extracted and GCS mRNA and β-actin mRNA levels were analyzed by reverse transcription PCR. B. Cell lysates were subjected to immunoblotting with anti-GCS and anti-actin antibodies. C. GCS activity was measured using NBD C6-ceramide as substrate as described under "Material and methods". The data represent means ± SEM of three values (***, $p<0.001$). D. Cultured cells were radiolabeled with [^{14}C]galactose for 24 h, lipids were extracted and then [^{14}C]GlcCer, [^{14}C]LacCer, [^{14}C]TrihexCer and [^{14}C]GM3 were resolved by TLC and quantified as described under "Material and methods". Endogenous species were detected with 3% cupric acetate − 8% phosphoric acid reagent and the results are represented by dashed bars. The data represent means ± SEM of three to five values. E. MDCK cells grown on coverslips were fixed and stained 48 h post-treatment with the apical marker gp135 (red) and the basolateral marker α-catenin (green). Cells were imaged by confocal microscopy with 0.5 μm z-sections. 3D-assembled confocal sections (top) and representative confocal xz section images (bottom). F. Cells were cultured and stained with Na/K-ATPase as basolateral marker (red), assembled images (xz) are shown. G. Cartoons of gp135 (red) and α-catenin (green) localization in control and GSL-depleted cells.

proteins. Cell viability was not affected by the treatments (data not shown). The images from hypertonicity-subjected cells transfected with scrambled siRNA and those treated with L-PDMP were similar, so representative images are shown as control. 3D-reconstructions and xz sections in Fig. 3E show that the treatment with D-PDMP caused the disappearance of the apical accumulation of gp135 in most of the cells, with the concomitant loss of the typical columnar cell shape with the apical dome characteristic of differentiated MDCK cells. Similarly, the knockdown of GCS caused a decrease in the apical gp135 fluorescent signal in some cells with the complete disappearance in other cell sets (arrowhead). Furthermore, treated monolayers had lower height than control ones, reaching an average lateral domain height of 6.58 μm in D-PDMP-treated cells and of 5.82 μm in GCS-knockdown cells *vs* 9.07 μm in control cells.

In order to verify the existence of an alteration in the lateral membrane domain, we also analyzed the distribution of Na/K ATPase, commonly used as a basolateral marker of epithelial cells [29]. Na/K-ATPase appeared only laterally located both in siRNA GCS and in D-PDMP-treated cells (Fig. 3F), thus demonstrating no alteration of the lateral domain.

These results suggest that GSL synthesis is specifically involved in the development of the differentiated apical domain but does not affect the lateral protein localization.

Overall, these experiments demonstrate that GSL synthesis plays a central role in the development of the differentiated apical domain of MDCK cells. A model is proposed in Fig. 3G.

3.5. Glucosylceramide synthase inhibition alters primary cilium formation in MDCK cells

The final stage in the process of epithelial cell differentiation is the outgrowth of a primary cilium from the apical surface [10]. The primary cilium is a hair-like structure that protrudes from the center of the apical membrane [9]. To test whether cultured MDCK cells subjected to hypertonicity developed this stage of differentiation, we analyzed the formation of the primary cilium by confocal immunofluorescence both with antiacetylated tubulin antibody, to reveal the cilium, and with anti Zo-1, a tight junction component commonly used as a reference to visualize the most apical section of lateral membrane [30]. As expected, Zo-1 perfectly delineated the periphery of the cells under both isotonic and hypertonic conditions (Fig. 4A). After 3 days of confluence, the isotonicity-cultured cells did not show cilium formation and the fluorescent signal for acetylated tubulin was very low and diffusely distributed, except in dividing cells, where acetylated tubulin formed the mitotic spindle (arrowhead). After subjecting to hypertonicity, approximately 30% of cells showed the primary cilium projecting from the apical surface (Fig. 4A, arrows), thus reflecting that hypertonicity in fact induced the final stage of the morphological differentiation of MDCK cells. In order to evaluate the involvement of GSLs in this process, we next tested the effect of the inhibition of GSL synthesis and the GCS knockdown on cilium formation. The number of ciliated cells was similar in hypertonicity-subjected cells either transfected with scrambled siRNA or treated with L-PDMP, so a representative photo is shown as hypertonicity. As seen in Fig. 4, GSL-reduced cells failed to develop the primary cilium, although the signal of acetylated tubulin appeared diffusely distributed in both GCS knockdown and D-PDMP-treated cells, demonstrating that tubulin acetylation was not affected by the inhibition of GSL synthesis. Quantitative analysis of ciliated cells showed that the number of ciliated cells was significantly decreased in GSL-reduced monolayers (Fig. 4B).

Taken together, the results demonstrate that, even under hypertonicity, MDCK cells cannot fully differentiate if GSL synthesis is impaired.

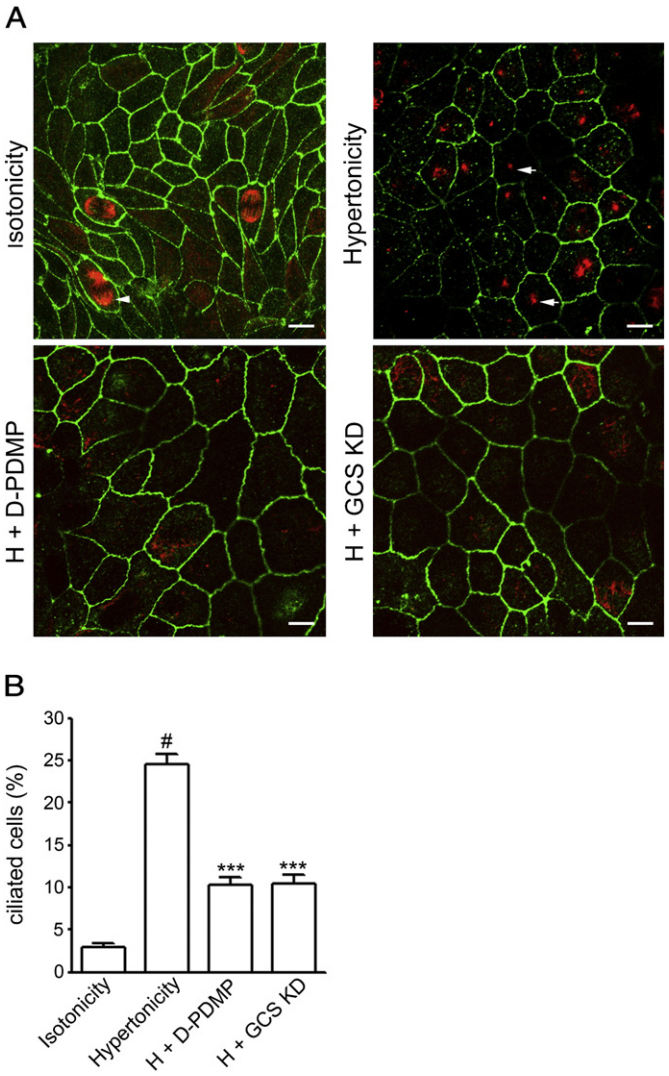

Fig. 4. Hypertonicity induces cilium formation in MDCK cells by a GSL-dependent mechanism. A. Confluent monolayers of MDCK cells were incubated under isotonic or hypertonic conditions or subjected to hypertonicity plus 5 μM D-PDMP (H + D-PDMP) or 400 nM GCS siRNA (H + GCS KD). Cells were stained for Zo-1 (green) and acetylated tubulin (red), and imaged by confocal microscopy. Bar, 10 μm. B. Quantification of the number of ciliated cells from the different monolayers. Data are means ± SEM of three independent experiments (#, $p<0.001$ *vs* isotonicity; ***, $p<0.001$ *vs* hypertonicity).

3.6. Hypertonicity induces the increase in GSL synthesis by the salvage pathway and the activation of an FB1-resistant ceramide synthase

Since the synthesis of GSLs in MDCK cells occurred both under isotonicity and under hypertonicity, we next wondered why MDCK cells require a hypertonic extracellular environment to trigger a GSL-dependent program of differentiation. We first explored the possibility that different sources of ceramide served as substrate for GlcCer. Ceramide can be synthesized either by the *de novo* synthesis or by the salvage pathway, which re-utilizes long-chain sphingoid bases. L-Cycloserine (CS) inhibits serine palmitoyl transferase (SPT), the initial step of the *de novo* pathway (29), and Fumonisin B1 (FB1), a potent inhibitor of ceramide synthases (CerS), is able to inhibit both the *de novo* and the salvage pathways of ceramide synthesis [31]. To determine the metabolic source of ceramide involved in the synthesis of GlcCer, we used [^{14}C]galactose as a radioactive precursor and CS or FB1 as metabolic inhibitors and we also determined the endogenous mass in each condition (Fig. 5). The results showed that in

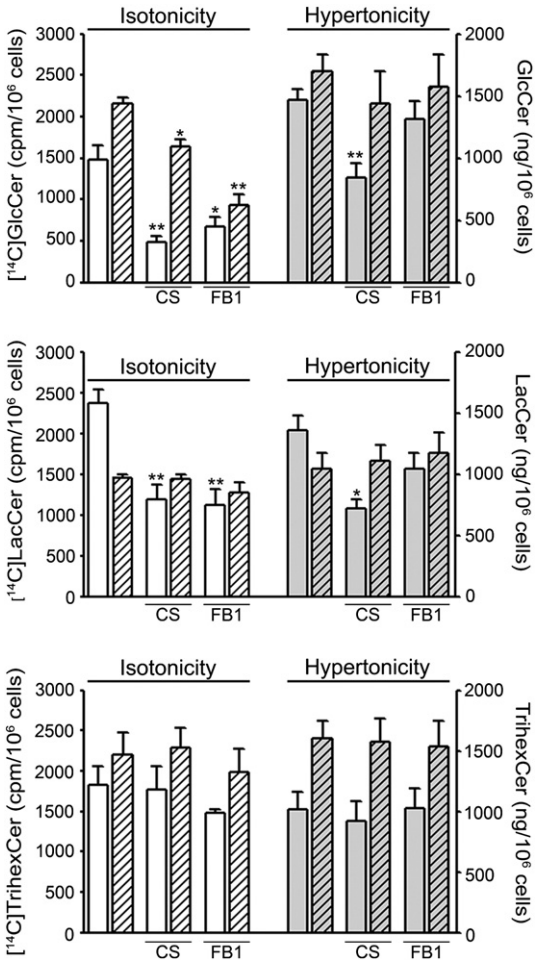

Fig. 5. Effect of the inhibition of sphingolipid synthesis in the metabolism of GlcCer, LacCer and TrihexCer in MDCK cells subjected to isotonic or hypertonic conditions. Confluent MDCK cells were radiolabeled for 24 h with [^{14}C]galactose under isotonicity (150 mM NaCl) or hypertonicity (300 mM NaCl) in the presence or absence of 0.5 mM L-cycloserine (CS) or 50 μM Fumonisin B1 (FB1) for 24 h. Radiolabeled lipids were extracted and quantified as described in "Material and methods". Endogenous GSLs were detected with 3% cupric acetate — 8% phosphoric acid reagent and the results are represented by dashed bars referenced to the right axis of the graph. The data represent mean ± SEM of three values (*, $p<0.05$; **, $p<0.01$).

isotonicity-cultured cells, both CS and FB1 significantly reduced [^{14}C]GlcCer (68 and 55% respectively) with decrease in the endogenous mass and also reduced [^{14}C]LacCer (50 and 53%) with no changes in the endogenous mass. Neither radiolabeled nor endogenous TrihexCer showed changes.

After 24 h of exposure to hypertonic medium, CS caused only a 42% decrease in [^{14}C]GlcCer and [^{14}C]LacCer with no reduction in their endogenous mass. No effect was observed in TrihexCer synthesis. The treatment with FB1 did not cause a significant decrease in the synthesis of GlcCer, LacCer or TrihexCer (Fig. 5).

These results indicate that, under isotonicity, GSL formation was mostly initiated by the CS-sensitive *de novo* pathway and mediated by FB1-sensitive CerS activity. Under hypertonicity, the inhibition of SPT was less effective in the blockage of GSL synthesis, which suggest the participation of the salvage pathway, and, more interestingly, insensitive to FB1, which could reflect the activation of an FB1-resistant CerS enzyme.

It is accepted that the incorporation of radiolabeled fatty acid precursor into SLs during short-term incubation is an indication of the *de novo* synthesis pathway, while long term incubation reflects the inclusion of the salvage pathway of SL synthesis [32]. Thus, to further investigate the pathway involved in the increased synthesis of GlcCer under hypertonic conditions, we compared the effects of high salt medium on [^{14}C]palmitic acid labeling of GlcCer under a pulse protocol *vs* a long-term incubation condition (Fig. 6A). If hypertonicity stimulates the *de novo* synthesis of GlcCer, then it should stimulate the acute incorporation of palmitate into GlcCer when cells are incubated with radiolabeled palmitic acid for 1 h. On the other hand, if hypertonicity stimulates the salvage pathway, steady-state labeling of GlcCer would be enhanced in response to high salt medium. Therefore, MDCK cells were subjected to hypertonicity and labeled with [^{14}C]palmitic acid for either 1 h or 24 h in the presence or absence of CS or FB1. Under the pulse protocol, hypertonicity did not enhance the incorporation of label into GlcCer, but when cells were labeled with [^{14}C]palmitic acid for 24 h, hypertonicity significantly induced increase in [^{14}C]GlcCer formation. Furthermore, the results in Fig. 6A show that sensitivity to CS and FB1 in isotonicity-cultured cells was different from that in hypertonicity-subjected cells. Under isotonicity, both CS and FB1 caused a clear decrease in [^{14}C]GlcCer. However, cells subjected to hypertonicity showed that [^{14}C]palmitic acid incorporation was only partially sensitive to CS and clearly resistant to FB1. These results indicate that the salvage pathway is induced in response to hypertonicity.

In order to find an explanation for the FB1 resistance of GSL synthesis, we further determined the activity of CerS, the current target enzyme of the inhibitor. To this end, the enzyme activity was measured by using [^{3}H]sphingosine as substrate in cells either subjected or not to hypertonicity and treated *in vivo* with FB1. As shown in Fig. 6B, under isotonic conditions, we detected a basal level of CerS activity, which was significantly inhibited by preincubation with FB1. The exposure to hypertonicity for 24 h induced a significant increase in CerS activity. Interestingly, instead of inhibiting it, FB1 caused a more than two-fold increase in CerS activity.

It is accepted the existence of various isoforms of CerS that have specificity for different fatty acids [33–37]. Venkataraman et al. have previously reported the existence of an FB1-resistant CerS in human embryonic kidney 293T cells transfected with uog 1, whose product ceramide is preferentially channeled to neutral GSLs enriched in stearoyl fatty acids [33]. In order to get any information about fatty acid specificity of the CerS activated by extracellular hypertonicity, we studied the incorporation of [^{14}C]stearic acid and [^{14}C]palmitic acid to ceramide under isotonicity and hypertonicity. As shown in Fig. 6C, hypertonicity induced an increase in ceramide labeled with both [^{14}C]stearic acid and [^{14}C]palmitic acid at 24 h of incubation with the radioactive precursors. FB1 significantly decrease the incorporation of both fatty acids to ceramide under isotonic condition, while neither [^{14}C]stearic acid nor [^{14}C]palmitic acid incorporation was inhibited by FB1 under hypertonicity. Therefore, hypertonicity induces the activation of a CerS insensitive to FB1 which could use both stearic acid and palmitic acid to synthesize ceramide. It is interesting to note that [^{14}C]stearic acid incorporation almost double that of [^{14}C]palmitic acid. This result is consistent with the previous observation of Venkataraman et al. [33], respect to the ceramide formed by a FB1-resistant CerS that is preferentially channeled to neutral GSLs enriched in stearoyl fatty acid.

Taken together the results suggest that hypertonicity induces the activation of an FB1-resistant CerS, whose product, ceramide, can serve as substrate for the synthesis of the GSLs required for MDCK cell differentiation.

3.7. The salvage pathway of GSL synthesis is involved in the differentiation of MDCK cells

To further characterize the effect of CS and FB1 on MDCK cell differentiation, we analyzed the distribution of the differentiation markers and the cilium formation by confocal immunofluorescence in hypertonicity-subjected cells incubated in the presence of the inhibitors. The 3D reconstructions in Fig. 7A show that MDCK cells

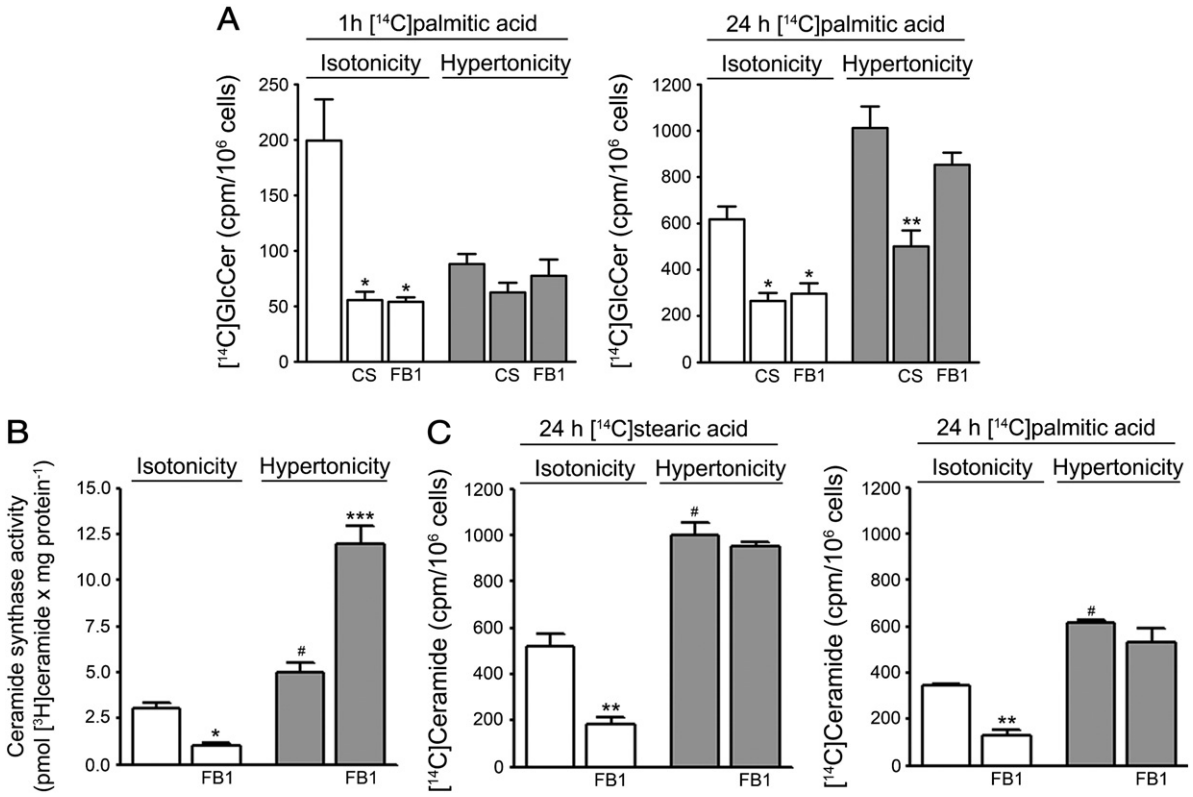

Fig. 6. Involvement of the salvage pathway in the increase in GlcCer synthesis induced by hypertonicity. A. Confluent MDCK cells were cultured under isotonicity (150 mM NaCl) or hypertonicity (300 mM NaCl) in the presence or absence of 0.5 mM L-cycloserine (CS) or 50 μM Fumonisin B1 (FB1) for 24 h and radiolabeled with [^{14}C]palmitic acid for 1 h or 24 h before harvesting. Radiolabeled lipids were extracted and quantified as described in "Material and methods". The data represent mean ± SEM of three values (*, $p<0.05$, **, $p<0.01$; *vs* control). B. For ceramide synthase activity determination, cells were treated for 24 h in the presence or absence of 50 μM FB1 and incubated in the presence of [^{3}H]sphingosine for 1 h as described under "Material and methods". Data shown are mean ± SEM of three independent experiments (*, $p<0.05$; *** $p<0.001$ *vs* control; #, $p<0.01$ *vs* isotonicity). C. For ceramide synthase characterization, cells treated or not with 50 μM FB1 as above were incubated in the presence of [^{14}C]stearic acid or [^{14}C]palmitic acid for 24 h as described under "Material and methods". Data shown are mean ± SEM of three independent experiments (**, $p<0.01$ *vs* control; #, $p<0.01$ *vs* isotonicity).

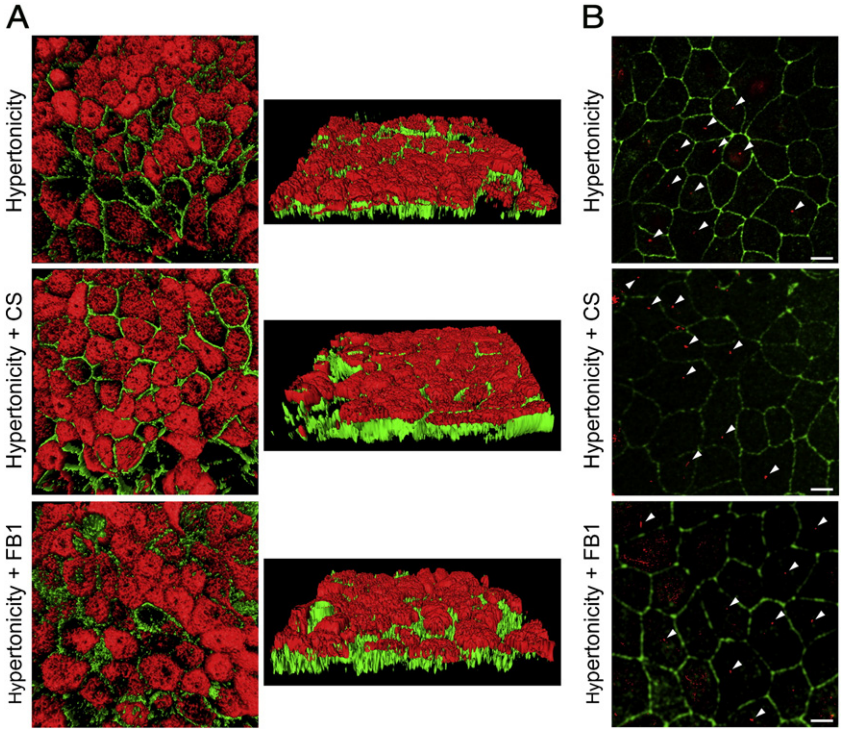

Fig. 7. Sphingolipid synthesis inhibition does not affect the MDCK cell differentiated phenotype induced by hypertonicity. A. Representative frontal (left) and dorsal (right) views of 3D-rendered confocal images of MDCK cell monolayers subjected to hypertonicity for 48 h (control) in the presence or absence of L-cycloserine (CS) or Fumonisin B1 (FB1), stained for α-catenin (green) and gp135 (red). B. Cells were cultured as in A, treated for 72 h, and stained for Zo-1 (green) and acetylated tubulin (red). Representative zoom sections are shown to visualize the ciliated cells (arrowheads). Bar, 10 μm.

developed the differentiated apical membrane domain reflected by gp135 accumulation on the apical surface, and that α-catenin perfectly outlined the cells, even in the presence of CS or FB1. Furthermore, neither CS nor FB1 treatment affected the development of the final stage of differentiation expressed by the cilium formation (Fig. 7B).

Taken together, these results demonstrate that the *de novo* pathway of ceramide synthesis is not involved in hypertonicity-induced MDCK cell final differentiation.

4. Discussion

In this study, we demonstrated that GSL synthesis defines hypertonicity-induced epithelial cell differentiation. The most direct evidence is provided by the observation that the inhibition of GCS prevented the development of the differentiated apical membrane in MDCK cells reflected by the impairment of apical gp135 accumulation and primary cilium formation.

MDCK is a cell line derived from the renal collecting duct epithelium, commonly used to study the process of cell differentiation. Differentiation is generally achieved by culturing cells on porous filters or by plating them on an artificial extracellular matrix such as collagen or Matrigel [9,38,39]. None of such protocols is able to cause physiological differentiation. The former has the inconvenience that when cells differentiate physiologically they do not have a porous surface to attach [40], whereas in the latter cells grow in media which are not their physiological extracellular matrix. Under our experimental conditions, MDCK cells secrete their own extracellular matrix, where they attach on their basal surface. On the other hand, external hypertonicity is a physiological extracellular environment of renal collecting duct cells required to maintain their differentiated phenotype [20,41].

In the present report we show for the first time that external hypertonicity is a sufficient condition to develop MDCK cell final differentiation, and that such process depends on GSL synthesis.

Under isotonicity, MDCK cells showed low level of polarity even after 48–72 h of confluence, as indicated by the presence of the apical marker protein gp135 in the lateral membrane. The hypertonic stress caused the disappearance of lateral gp135 and initiated the differentiation program, as reflected by apical accumulation of gp135 and primary cilium development. The acquisition of the fully differentiated phenotype was specifically impaired by the pharmacological inhibition of GSL synthesis and by the silencing of GCS gene expression. Although the inhibition of GSL synthesis caused a decrease in apical gp135 accumulation and the absence of cilium formation, it appears that the cells kept a polarized phenotype, since no gp135 was visible at the lateral membrane, and also, the lateral membrane markers α-catenin and Na/K-ATPase did not delocalize and remained mostly outlining the cells. These results demonstrate that the synthesis of GSLs is necessary for apical domain biogenesis but not for basolateral sorting of proteins.

Several lines of evidences have suggested, although not directly proved, the participation of GSLs in the differentiation of MDCK cells. Vieira et al. have shown that FAPP2, recently described as a specific GlcCer transfer protein [42], plays a specific role in apical transport in MDCK cells [43]. By using the matrigel model of MDCK cell differentiation, Vieira et al. further reported that the depletion of FAPP2 impairs the outgrowth of the primary cilium as well as the apical gp135 accumulation [11]. Recently, Halter et al. described that FAPP2 can transport newly synthesized GlcCer to the plasma membrane or via endoplasmic reticulum to enter the secretory pathway to be converted in LacCer [25]. Taking into consideration those previous reports, we hypothesize that the impairment of MDCK cell differentiation caused by FAPP2 depletion reported by Vieira et al. could be due to a failure in GlcCer availability. Thus, our finding that inhibition of GlcCer synthesis prevented MDCK cell differentiation can provide the link between those previous observations, and allow us to suggest that GCS is an upstream key enzyme for the initial development of the differentiated apical membrane.

In agreement with our results, the involvement of GSL synthesis in the polarized distribution of basolateral and apical proteins has been earlier reported by Mays et al. [44]. These researchers concluded that the basolateral sorting of Na/K-ATPase was not dependent on GSL synthesis, while the apical sorting of GPI-anchored proteins was closely related to GSLs. In such pioneer study, the authors found that the treatment with FB1 did not affect the apical delivery of gp135, the prominent apical protein in MDCK cells, and concluded that gp135 corresponds to a class of protein whose apical sorting is independent of GSL synthesis. We showed that FB1 did not affect the apical accumulation of gp135 but that both D-PDMP and the knockdown of GCS impaired gp135 accumulation, thus demonstrating the dependence on GSL synthesis. FB1 has been described as a potent inhibitor of CerS [23], an upstream enzyme along the pathway of GlcCer synthesis, and many of the studies on the biosynthesis of SLs have relied on the ability of FB1 to inhibit CerS. However, the results from this study clearly show that hypertonicity activates an FB1-insensitive CerS leading to a specific pool of ceramides, which further serve as precursors for the synthesis of GlcCer.

Ceramide can derive either from the *de novo* synthesis pathway initiated by condensation of serine and palmitoyl-CoA by the action of SPT or from the salvage pathway generated by hydrolysis of complex sphingolipids [31]. The results from the present study show that the pathway involved in hypertonicity-induced MDCK cell differentiation is the salvage but not the *de novo* pathway. We reached this conclusion because the inhibition of SPT did not affect the apical gp135 accumulation and the primary cilium formation.

Notably, Hanada et al. [45] reported the existence of two different pools of ceramide for GlcCer synthesis: a serine-derived one (from the *de novo* pathway), which is transported by the ceramide transport protein (CERT) to the GCS located in the trans Golgi, and a sphingosine-derived (from the salvage pathway) and CERT-independent one, transported preferentially to cis-medial Golgi. Probably, this latter pathway is the one involved in the process of MDCK cell differentiation and may constitute a physiological evidence of how a specific pathway of ceramide generation can define a specific function as is the triggering of epithelial cell differentiation.

Previous observations serve as a platform for the interpretation of our results. Venkataraman et al. found the induction of an FB1-resistant CerS whose product ceramide is preferentially channeled to GSLs synthesis in human embryonic kidney 293T cells transfected with uog 1 (a mouse homolog of LAG1, a CerS). Moreover, consistent with our results, the authors reported that the transfection with uog 1 produced increase in GlcCer, LacCer and TrihexCer but not GM3 [33]. On the other hand, Meivar-Levy and Futerman reported up-regulation of neutral GSL synthesis upon long-term treatment of 3T3 fibroblasts with FB1, although they did not evaluate CerS activity, which was probably increased [46]. Finally, more recently, Liu et al. reported that ceramide can induce the expression of GCS [47]. We consider that, under the metabolic point of view, the importance of the present report is that we provide evidence of the physiological activation of a coordinated pathway of GSL synthesis which includes CS-insensitive, FB1-upregulated ceramide synthesis that channels into neutral GSL synthesis. It is possible that the ceramide produced by the increased activity of the FB1-resistant CerS operates as an inducer of GCS, with the consequent increase in GSL synthesis. To our knowledge, the induced activation of an FB1-resistant CerS plays a central role in the process of differentiation. However, we want to emphasize that, although relevant, the increase in ceramide synthesis was not sufficient to induce cell differentiation because the inhibition of GCS was necessary to prevent cell differentiation.

It is accepted that extracellular hypertonicity is a physiological condition for the maintenance of the differentiated phenotype of renal collecting duct cells. Here, we demonstrated that extracellular

hypertonicity is sufficient to induce the morphological differentiation of MDCK cells. Furthermore, we demonstrated that this process occurs by displaying a sphingolipid metabolic program that includes the formation of a specific pool of GSLs. The results suggest as precursor a specific pool of ceramides formed by the activation of an FB1-resistant CerS as a component of the salvage pathway.

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