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Bile acids modulate the Golgi membrane fission process via a protein kinase Cη and protein kinase D-dependent pathway in colonic epithelial cells

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Deoxycholic acid (DCA) is a secondary bile acid that modulates signalling pathways in epithelial cells. DCA has been implicated in pathogenesis of colon carcinoma, particularly by activation of the protein kinase C (PKC) pathway. Ursodeoxycholic acid (UDCA), a tertiary bile acid, has been observed to have chemopreventative effects. The aim of this study was to investigate the effect of DCA and UDCA on the subcellular localization and activity of PKCη and its downstream effects on Golgi structure in a colon cancer cell model. PKCη expression was localized to the Golgi in HCT116 colon cancer cells. DCA induced fragmentation of the Golgi in these cells following activation of PKCη and its downstream effector protein kinase D (PKD). Pretreatment of cells with UDCA or a glucocorticoid, dexamethasone, inhibited DCA-induced PKCη/PKD activation and Golgi fragmentation. Knockdown of glucocorticoid receptor (GR) expression using small interfering RNA or inhibition using the GR antagonist mifepristone attenuated the inhibitory effect of UDCA on Golgi fragmentation. Elevated serum and faecal levels of DCA have been previously reported in patients with ulcerative colitis (UC) and colon cancer. Analysis of Golgi architecture *in vivo* using tissue microarrays revealed Golgi fragmentation in UC and colorectal cancer tissue. We have demonstrated that DCA can disrupt the structure of the Golgi, an organelle critical for normal cell function. Inhibition of this DCA-induced Golgi fragmentation by UDCA was mediated via the GR. This represents a potential mechanism of observed chemopreventative effects of UDCA in benign and malignant disease of the colon.

Introduction

Deoxycholic acid (DCA) is the predominant secondary bile acid implicated as a tumour promoter in colon carcinogenesis (1). Diets rich in saturated fats are linked to excessive bile acid production and associated with increased risk of progression to colon cancer (2,3). Long-term ulcerative colitis (UC) is also a risk factor for colon cancer and serum and faecal levels of DCA are elevated in patients both with UC and colon cancer (4). DCA can modulate cell signalling pathways and activity of transcription factors, such as activator protein-1 and nuclear factor-kappaB (NF-κB), shown previously to be involved in tumour progression (5). NF-κB activity is increased in most human cancers (6), whereas activator protein-1 shows increased activity in transformed cell lines and its transactivation is required for tumour promotion *in vivo* (7,8). DCA activated signalling pathways associated with tumourigenesis, including extracellular signal-regulated kinase (activated protein kinaseERK)/mitogen-activated protein kinase (MAPK)-, protein kinase B/AKT- and protein kinase C (PKC)-related pathways (9,10). The conventional and novel members of the PKC family are receptors for the tumour-promoting phorbol esters, such as phorbol 12-myristate 13-acetate. PKC expression and activity have been implicated in colon cancer promotion as it is a key regulator of proliferation in colonic epithelium (11). PKC activity has been associated with the amount and type of dietary fat where a high-fat diet containing corn oil increased colonic mucosal levels of PKC activity in a rat model (12). We have shown previously DCA-mediated activation and translocation of PKC isoenzymes implicated in tumour progression, including translocation of PKCα, PKCδ, PKCε and PKCβ1 in HCT116 colon cancer cells (13). PKCη is predominantly expressed in epithelial cells where it is localized to the Golgi apparatus and is involved in differentiation. PKCη is expressed in suprabasal layers in the intestine where cell differentiate but not in the basal layer where cells divide (14). Any change in its expression/activation would result in altered cell differentiation in the colon that potentially results in neoplastic progression. PKCη may also play a role in carcinogenesis as several studies have looked at expression levels of PKC in human colon cancer tissue and found altered levels of PKCη expression in tumour versus normal tissue (15–17). PKCη also promotes production of the pro-inflammatory cytokine interleukin-6 (18) that is up-regulated in the pre-neoplastic colon cancer diseases (19). PKCη is also important in epithelial tight junction regulation (20).

The Golgi apparatus is the organelle responsible for transport, sorting and processing of proteins from the endoplasmic reticulum. Proteins enter the *cis*-Golgi network and are sorted and packaged into carrier vesicles at the *trans*-Golgi network (TGN) ready to be transported to the plasma membrane, endosomes and endoplasmic reticulum. These carriers are dissociated from the TGN by a controlled process termed membrane fission. Once cargo accumulates at the TGN, a G-protein-coupled receptor activates a trimeric G-protein at the TGN resulting in the activation of PKCη and recruitment and phosphorylation of protein kinase D (PKD) (by PKCη). PKD then activates downstream targets to control membrane carrier fission (21). A natural marine product, ilimaquinone, overactivates the fission machinery, resulting in complete vesiculation of the Golgi via a PKD-dependent process (22–24). Golgi fragmentation, which is associated with abnormal protein processing, has been observed in the MCF-7 breast tumour cell line and also in SW480, Caco-2, HT-29 and T-84 colon cancer cell lines. In certain cases, the cause of Golgi fragmentation is thought to be associated with altered Golgi pH (25); however, factors involved in the regulation of this phenotype *in vivo* are not characterized.

Ursodeoxycholic acid (UDCA) is a tertiary bile acid traditionally used for the treatment of hepatobiliary disease, including primary biliary cirrhosis, primary sclerosing cholangitis (PSC) and gallstones (26). It is a hydrophilic non-toxic bile acid and has been shown to be cytoprotective against other more hydrophobic bile acids, such as DCA (27). We have previously demonstrated that UDCA can prevent DCA-induced translocation of PKCα, PKCδ, PKCε and PKCβ1 (13) and inhibit both cytokine- and DCA-induced activation of NF-κB and activator protein-1 (5). The therapeutic effects of UDCA have been attributed to several mechanisms including modulation of classical mitochondrial pathways of apoptosis (28) and its interaction with the glucocorticoid receptor (GR) (29,30). UDCA has immunomodulatory and anti-inflammatory effects similar to glucocorticoids and has been shown to suppress NF-κB-dependent transcriptional activity (31) and interferon-γ-induced major histocompatibility complex class II gene expression via a GR-dependent pathway (30).

The aim of this study was to investigate the effect of DCA and UDCA on the Golgi membrane fission process in colonic epithelial cells. We demonstrate that DCA induced activation of PKCη and consequently, PKD, resulting in fragmentation of the Golgi in

Abbreviations: CA, constitutively active; DAG, diacylglycerol; DEX, dexamethasone; DCA, deoxycholic acid; GFP, green fluorescent protein; GR, glucocorticoid receptor; MFT, mifepristone; NF-κB, nuclear factor-kappaB; PKC, protein kinase C; PKD, protein kinase D; PSC, primary sclerosing cholangitis; siRNA, small interfering RNA; TGN, trans-Golgi network; UC, ulcerative colitis; UDCA, ursodeoxycholic acid.

HCT116 colon cancer cells. This DCA-stimulated Golgi fragmentation can be inhibited by the hydrophilic bile acid, UDCA, in a GR-dependent manner. In a cellular context, fragmentation of the Golgi has profound implications for protein processing and may result in alterations to processes, such as signal transduction, cellular proliferation and cell–cell adhesion, all key elements of carcinogenesis. We further demonstrate increased Golgi fragmentation in tissue from UC and colon cancer patients when compared with that from normal controls.

Materials and methods

Cell culture and reagents

HCT116 colon cancer cells were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in McCoy's 5A medium supplemented with 10% (vol/vol) foetal bovine serum (Gibco BRL, Grand Island, NY). DCA, UDCA, dexamethasone (DEX), mifepristone (MFT) and dimethyl sulphoxide were all obtained from Sigma–Aldrich Chemical Co. (St Louis, MO). DCA, UDCA and MFT were solubilized in dimethyl sulphoxide, whereas DEX was dissolved in ethanol (100% vol/vol; BDH, Dorset, UK). PKCη plasmids were a gift from the late Dr F.J.Johannes (Institute of Cell Biology and Immunology, University of Stuttgart, Germany). Transient transfections were conducted using GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA), Fugene HD (Roche Diagnostics, Basel, Switzerland) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturers protocols. Experiments were performed 24 h after transfection (or in some cases cells were pretreated with UDCA for 1 h prior to transfection). A Smartpool of pre-designed small interfering RNA (siRNA) oligos (ON-TARGET plus siRNA reagents) targeting the GR (siGR), scrambled control siRNA (siControl) and siRNA transfection reagent were purchased from Dharmacon (Lafayette, CO). Cells were seeded and transfected with 100 nM siGR or siControl for 24 h. Appropriate wells were pretreated with 300 μM UDCA and cells were left for a further 24 h prior to treatment with 300 μM DCA for 6 h.

Western blot analysis

Knockdown of the GR by siRNA was confirmed by western blot analysis. To investigate activation of PKD and PKCη by bile acids, lysates of stimulated cells were prepared and analysed using anti-phospho-PKD (Ser 744/748), anti-PKD (Cell Signalling Technology, Danvers, MA), anti-phosphospecific-PKCeta (pT655) (Biosource, Nivelles, Belgium) or anti-PKCη (Santa Cruz Biotechnology, Santa Cruz, CA).

Gaussia luciferase secretion assay

To determine the effects of DCA on protein secretion, we used a Gaussia luciferase assay (New England Biolabs, Ipswich, MA). Cells were transfected with a Gaussia luciferase construct using Fugene HD (Roche Diagnostics) according to the manufacturers protocol. Twenty-four hours post-transfection, cells were treated with Brefeldin as positive control, vehicle control (dimethyl sulphoxide) or DCA at indicated concentrations for either 1 or 6 h. The amount of luciferase protein secreted by the cells was quantified by measuring luminescence.

Tissue microarray construction

For all cases, haematoxylin- and eosin-stained slides from formalin-fixed paraffin-embedded tissue blocks were used to identify colonic mucosa. These areas were aligned with the tissue block and four 6 mm cores taken and transferred to a recipient block using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD). Four micrometre sections were cut for immunofluorescence studies and mounted onto SuperFrost Plus adhesive slides (Menzel-Glaser, Braunschweig, Germany).

Immunofluorescent staining

Cells were fixed with 4% paraformaldehyde and then permeabilized with 0.1% (vol/vol) Triton X-100/phosphate-buffered saline followed by blocking with 5% bovine serum albumin/phosphate-buffered saline. Cells were incubated with anti-Golgi 58K protein or anti-GM130 Golgi antibodies (Sigma–Aldrich Chemical Co.) and then incubated with AlexaFluor-488-conjugated secondary antibody (Invitrogen).

Quantification of Golgi fragmentation using high-content analysis

The GE Incell-1000 is a microscope-based screening platform capable of large-scale objective analysis of fluorescently labelled cells using automated image acquisition, data management and multiparametric analysis. For analysis of Golgi fragmentation, cells were treated in 96-well plates and stained for Golgi using a GM130 antibody as outlined above. Six fields of view per well were acquired using a ×20 objective in duplicate wells for $n = 3$ experiments. Fragmentation was measured using the Investigator software package (GE Healthcare, Piscataway, NJ) that uses an algorithm specific for detection of objects within the cell. The multi-target analysis algorithm was optimized to detect objects (Golgi fragments) within a cell using untreated cells with intact Golgi as a negative control and Brefeldin-A (1 μg/ml)-treated cells as positive control for Golgi fragmentation (32). The 'object mean area' parameter was used to classify cells as having intact Golgi (object mean area of >0.5 μm) or fragmented Golgi (object mean area of <0.5 μm) with up to 300 cells analysed per treatment group. Examples of cellular analysis are provided as supplementary material (supplementary Figure 1 is available at *Carcinogenesis* Online).

Patient tissue collection and analysis

In order to assess the clinical significance of Golgi fragmentation, we examined Golgi structure in tissues from five tissue types: (i) normal individuals ($n = 32$, 32 biopsies from 32 patients with no history of malignant disease); (ii) patients with UC without dysplasia ($n = 10$, 27 biopsies from 10 UC patients with no progression to dysplasia); (iii) patients with UC with dysplasia ($n = 6$, 18 biopsies from six UC patients with progression to dysplasia); (iv) UC-associated cancer ($n = 2$, 16 biopsies from two UC patients with progression to cancer) and (v) sporadic colonic cancer ($n = 22$, 22 biopsies from 22 patients). Normal individuals are defined as patients undergoing colonoscopy for investigation of altered bowel habit but whose histological investigations were normal and haematological indices and biochemical inflammatory markers (erythrocyte sedimentation rate and C-reactive protein) were normal. All patients were recruited by the Centre for Colorectal Disease (by D.O.D). Following endoscopy or surgery, tissues were fixed in 1% formalin and embedded in paraffin. Ethical approval was granted by the St Vincent's Hospital Ethics and Medical Research Committee to conduct this study. Tissue microarrays were stained with a TGN46 Golgi antibody. This antibody gave superior staining in paraffin-embedded tissue sections compared with either the GM130 or the 58K Golgi antibodies. A simple scoring system was devised to describe Golgi fragmentation with 0 corresponding to no Golgi fragmentation, 1 to describe partial fragmentation and 2 to describe complete Golgi fragmentation. Examples of these are included (supplementary Figure 2 is available at *Carcinogenesis* Online). The median and interquartile ranges were used for statistical analysis. Samples were coded and all stages of examination were performed blind to the tissue status.

Statistical analysis

Statistical comparison between groups for *in vitro* data was carried out using analysis of variance with least significant difference post hoc correction to examine differences between groups. Data are graphically represented as the mean ± SEM. For human tissue analyses, continuous data are presented as median and interquartile ranges. Non-parametric data were assessed using the Kruskal–Wallis test and Mann–Whitney *U*-test where appropriate. All *P*-values are two sided and *P*-values <0.05 were considered statistically significant in all analyses. All data were analysed using the SPSS™ statistical software package (SPSS, Chicago, IL).

Results

DCA altered the subcellular localization of PKCη and induced Golgi fragmentation in HCT116 colon cancer cells

DCA (300 μM) was shown previously to induce translocation of PKCα, PKCβ$_1$, PKCε and PKCδ, whereas UDCA prevented DCA-induced activation of these PKC isoenzymes (13). To investigate the effect of DCA on cellular localization of the novel PKCη isoform, HCT116 cells were transfected with PKCη-green fluorescent protein (GFP) and then treated with DCA for 1, 6 or 18 h (Figure 1A). In resting cells, PKCη was localized in a membranous stack adjacent to the cell nucleus. As PKCη was shown previously to be associated with the Golgi (15), we also stained cells using an anti-58K Golgi marker and found it colocalized with PKCη. DCA caused dispersal of the Golgi and the associated PKCη-GFP in a vesicular pattern throughout the cytoplasm demonstrating that DCA induced Golgi fragmentation in these cells. This DCA-induced Golgi fragmentation was observed over a concentration range between 50 and 300 μM (Figure 1B).

DCA-induced Golgi fragmentation was reversible

To determine whether DCA-induced Golgi fragmentation was reversible (and not an irreversible pro-apoptotic event), HCT116 cells were treated with 300 μM DCA for 6 h and then washed with phosphate-buffered saline and medium was replaced. Cells were fixed with 4%

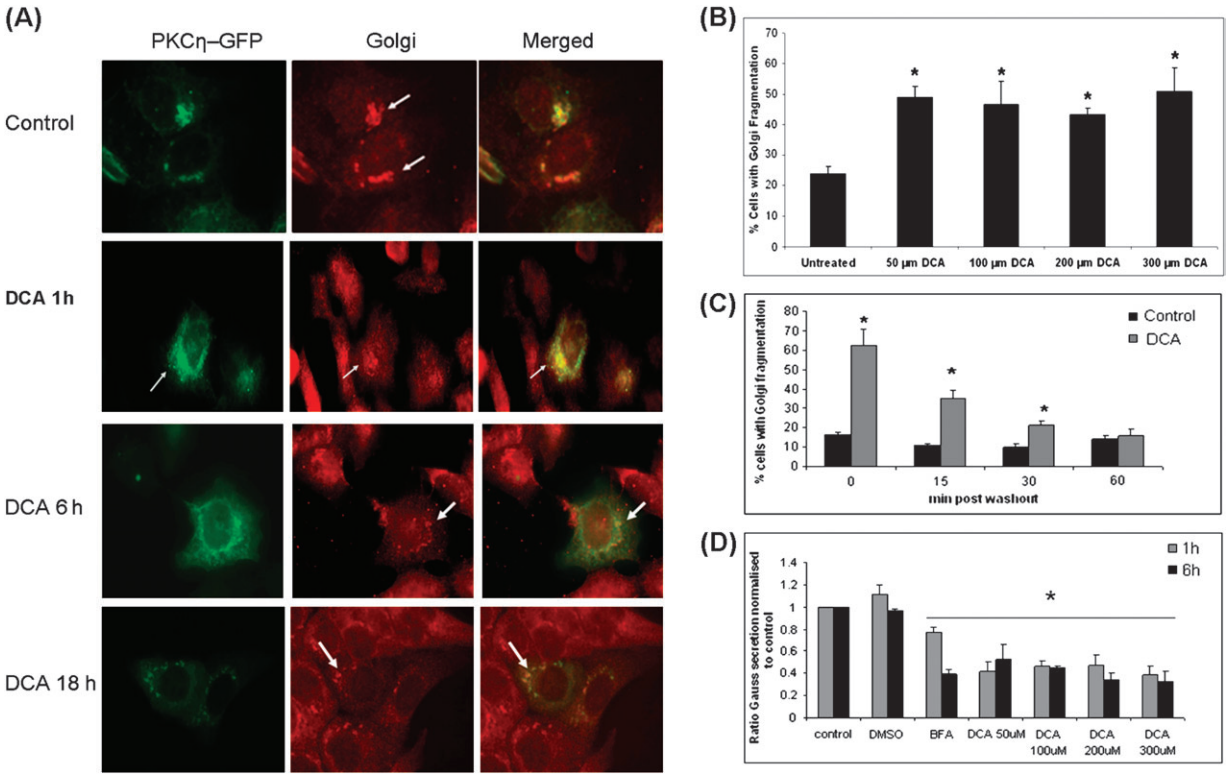

Fig. 1. DCA disrupted PKCη localization and induced Golgi fragmentation and impaired Golgi function in HCT116 cells. (**A**) Cells were transfected with PKCη-GFP (green) and then treated with 300 μM DCA for 6 or 18 h. Golgi were identified using a p58K antibody (red, indicated by white arrow) and visualized using a Nikon T800 fluorescent microscope. Original magnification ×40. (**B**) Cells were treated with 50, 100, 200 or 300 μM DCA for 6 h. Golgi were identified using the GM130 antibody. The percentage of cells with fragmented Golgi was quantified using the Incell-1000 and Investigator software package. (**C**) HCT116 cells were treated with 300 μM DCA or vehicle (control) for 6 h and then washed with phosphate-buffered saline and medium was replaced. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline at the timepoints indicated. Golgi fragmentation was quantified as in (B). (**D**) The secretory capacity of cells after treatment with DCA was assessed using a Gaussia luciferase assay. Cells were transfected with a Gaussia luciferase construct and 24 h post-transfection, cells were treated with Brefeldin-A (BFA) as positive control, vehicle control [dimethyl sulphoxide (DMSO)] or DCA at indicated concentrations for either 1 or 6 h. The amount of luciferase protein secreted by the cells was quantified by measuring luminescence. All data were expressed as the mean ± SEM and analysed by analysis of variance with least significant difference post hoc correction ($^{*}P < 0.05$ versus control).

paraformaldehyde at various timepoints up to 1 h as indicated in Figure 1C. The percentage of cells with fragmented Golgi was quantified by high-content analysis. Consistent with visual inspection, at 6 h post-treatment, DCA induced Golgi fragmentation in 62.3 ± 8.5% cells compared with fragmentation in 16.2 ± 1.4% of untreated cells. Following removal of DCA, the Golgi reformed in a time-dependent manner. The percentage of cells with fragmented Golgi 60 min post-washout of DCA (16.0 ± 3.2%) was the same as untreated cells (14.3 ± 1.7%). In addition, cell viability was assessed using the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay and by the propidium iodide exclusion assay. No significant changes in cell viability were observed using the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay compared with untreated control, whereas those observed using propidium iodide were low (5.8 ± 1.7%) at the 6 h time period for 300 μM DCA (supplementary Figure 3 is available at *Carcinogenesis* Online).

DCA-induced Golgi fragmentation impairs protein secretion

To test the functional significance of the observed DCA-induced Golgi fragmentation, the secretory capacity of cells after treatment with DCA was assessed using a Gaussia luciferase assay. There was a significant decrease in secretion of luciferase into the supernatant in DCA-treated cells compared with controls (Figure 1D).

DCA induced phosphorylation and activation of PKCη and PKD

To investigate whether DCA overactivates the membrane fission process, we examined the effect of DCA on PKCη and PKD activation in HCT116 cells. Activation of PKCη is associated with phosphorylation on Ser 729 and PKD with phosphorylation on Ser 744/748. HCT116 cells were cultured in 0.5% serum for 24 h prior to the experiment. Cells were treated with 300 μM DCA for 0, 15, 30 or 60 min. Cell lysates were prepared and PKCη and PKD phosphorylation was assessed by western blot analysis (Figure 2A). DCA induced phosphorylation and therefore, activation of PKCη and PKD at 15, 30 and 60 min. To confirm the involvement of PKCη in DCA-induced Golgi fragmentation, we transfected cells with a GFP-tagged PKCη-kinase dead construct (PKCη-KD-GFP) followed by treatment with 300 μM DCA for 6 h. The number of cells expressing the PKCη-kinase dead (PKCη-KD) construct with fragmented Golgi in response to DCA treatment was significantly reduced compared with those not expressing the mutant enzyme (Figure 2B).

Pretreatment of HCT116 cells with UDCA or DEX inhibits PKCη activation and Golgi fragmentation

As we previously showed that UDCA could inhibit DCA-induced translocation of other PKC isoforms (13), we investigated if it could inhibit DCA-mediated modulation of PKCη. HCT116 cells were transfected with PKCη-GFP, pretreated with 300 μM UDCA for 1 h and then exposed to 300 μM DCA for 18 h. UDCA inhibited DCA-induced Golgi fragmentation and associated PKCη dispersal (Figure 3A).

Constitutively active (CA) PKCη was shown previously to cause Golgi fragmentation in HeLa cells (33). To determine whether UDCA or DEX can directly target this process, HCT116 cells were treated with either 300 μM UDCA or 10 nM DEX for 1 h prior to transfection

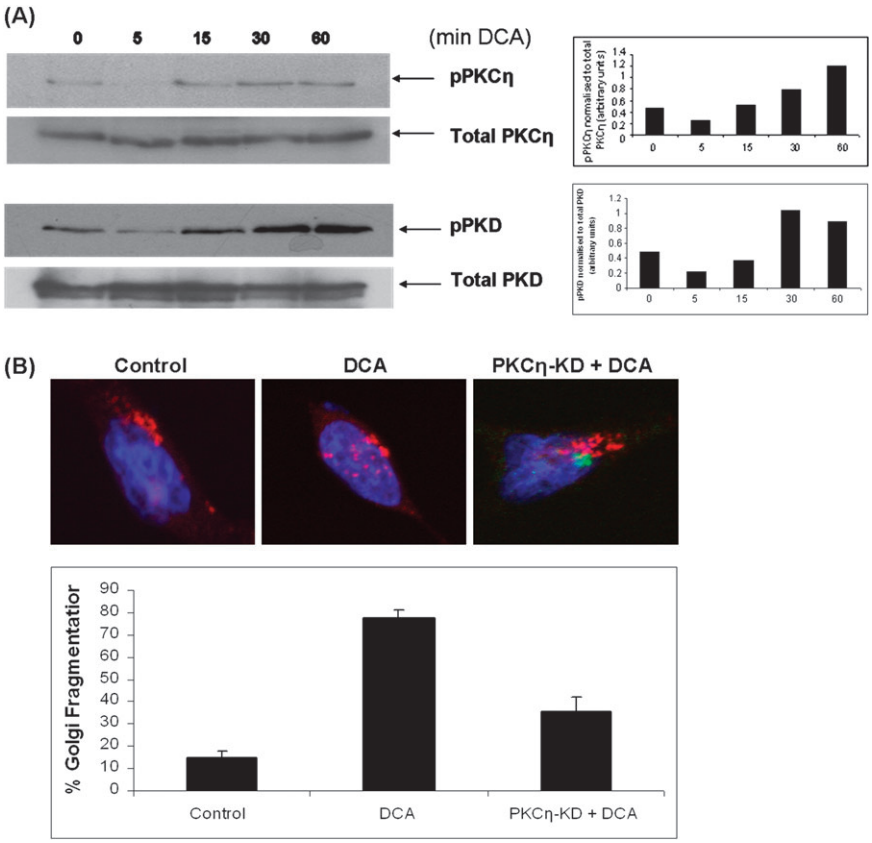

Fig. 2. DCA induced activation of PKCη and PKD in HCT116 cells. DCA could no longer induce Golgi fragmentation in cells transfected with PKCη-kinase dead (PKCη-KD) construct. (**A**) HCT116 cells were cultured in 0.5% serum for 24 h prior to stimulation for the timepoints indicated with 300 μM DCA. Cell lysates were prepared and pPKCη, total PKCη, pPKD or total PKD expression was assayed by western blot using antibodies specific to phospho-PKCη (Biosource), total PKCη (Santa Cruz Biotechnology), phospho-PKD (Ser 744/748) or total PKD (Cell Signalling Technology). Graphs of densitometry measurements are included. (**B**) HCT116 cells were transfected with PKCη-KD-GFP (green) and 24 h post-transfection, cells were treated with 300 μM DCA for 6 h. Cells were fixed and Golgi were identified using GM130 (red) antibody. Nuclei were stained with Hoechst (Invitrogen). At least 100 cells were counted for each group (control, DCA treated and DCA treatment following PKCη-KD transfection). Slides were coded and quantification was performed blind to treatment.

with a CA PKCη (stained in red). Golgi were visualized by immunofluorescence using the anti-58K Golgi antibody (green, Figure 3B). Cells transfected with CA PKCη had fragmented Golgi. Pretreatment with DEX or UDCA 1 h prior to transfection prevented CA PKCη-induced fragmentation.

UDCA displays structural similarity to glucocorticoids, exerts anti-inflammatory effects and was shown previously to activate the GR in primary rat hepatocytes (34). Therefore, the effects of DEX, a synthetic glucocorticoid, and UDCA on DCA-induced Golgi fragmentation were compared (Figure 3C). To confirm hyperactivation of the fission machinery and complete fragmentation of the Golgi, an antibody targeting a protein resident in the *cis*-Golgi network GM130 was used to examine the effect on Golgi morphology. DCA caused complete vesiculation of the Golgi structure. Both UDCA and DEX inhibited DCA-induced Golgi fragmentation.

UDCA or DEX pretreatment prevented DCA-induced PKD phosphorylation

PKD is necessary for the detachment (fission) of transport carriers from the TGN. As DCA activated PKD, we investigated if UDCA or DEX could prevent DCA-mediated activation of PKD. HCT116 cells were pretreated with UDCA (300 μM) or DEX (10 nM) for 1 or 2 h prior to treatment with DCA (300 μM) for 2 h. Cell lysates were prepared and PKD phosphorylation (pPKD) was assessed by western blot (Figure 4). Pretreatment with either UDCA or DEX prevented DCA-induced activation of PKD.

The GR is required for UDCA-mediated inhibition of DCA-induced Golgi fragmentation

Since the synthetic glucocorticoid DEX exerted the same effects as UDCA, inhibiting (i) DCA-induced Golgi fragmentation; (ii) CA PKCη-induced Golgi fragmentation and (iii) DCA-induced PKD activation, the potential involvement of the GR in these processes was investigated. When GR expression was knocked down using siRNA or when the GR was blocked using the GR antagonist, MFT, UDCA-mediated inhibition of DCA-induced Golgi fragmentation was attenuated (Figure 5A–C). There was no difference in Golgi fragmentation in cells treated with UDCA (33.8 ± 3.1%) or MFT (34.7 ± 3.3%) alone compared with untreated control cells (33.3 ± 2.2%). DCA induced Golgi fragmentation in 54.4 ± 1.2% cells. Pretreatment with UDCA inhibited DCA-induced fragmentation (42.9 ± 4.4%). UDCA could no longer overcome DCA-induced fragmentation when cells were treated with MFT (57.4 ± 3.7%) or when the GR was knocked down using siRNA (51.7 ± 4.1%), suggesting that UDCA mediates its protective effects via the GR in this system.

Golgi fragmentation is evident in vivo in patients with UC and colon cancer

To investigate whether the DCA-mediated effect on Golgi architecture observed in HCT116 cells may also be seen *in vivo* in UC (inflammatory) or cancer, Golgi structure was examined in five tissue types (i) normal; (ii) UC without dysplasia; (iii) UC with dysplasia; (iv)

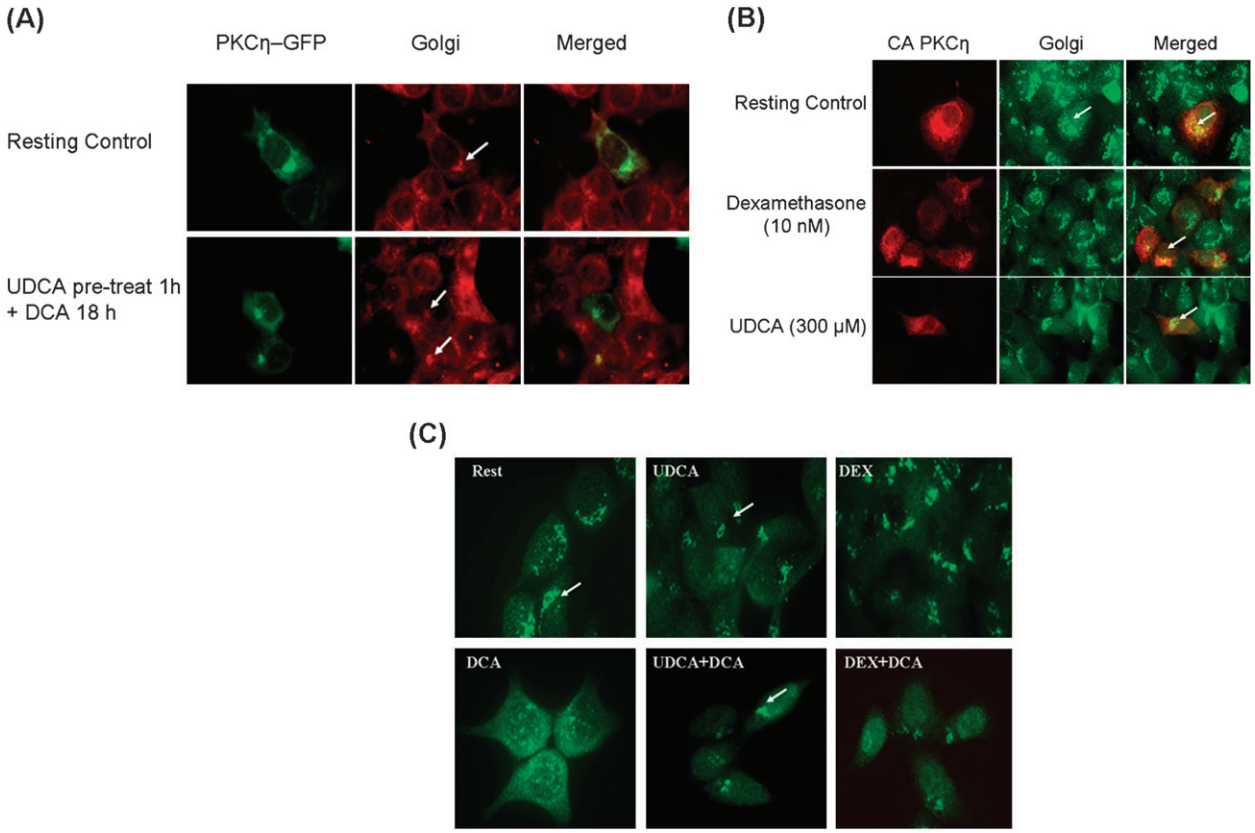

Fig. 3. UDCA and DEX inhibit both DCA-induced and PKCη-induced Golgi fragmentations. (**A**) UDCA inhibits DCA activation of PKCη. HCT116 cells were transfected with PKCη-GFP (green) or pretreated with 300 μM UDCA for 1 h followed by treatment with 300 μM DCA for 18 h. UDCA inhibited DCA-induced PKCη translocation and Golgi fragmentation (red, indicated by white arrow) throughout the cytoplasm. (**B**) DEX or UDCA inhibits CA PKCη-induced Golgi fragmentation. HCT116 cells were transfected with a CA PKCη (red) or treated with either 10 nM DEX or 300 μM UDCA for 1 h prior to transfection. (**C**) UDCA and DEX inhibit DCA-induced Golgi fragmentation. Cells were treated with 300 μM DCA, 300 μM UDCA or 10 nM DEX for 6 h or pretreated with 300 μM UDCA for 18 h or 10 nM DEX for 1 h followed by treatment with 300 μM DCA for 6 h. Golgi were identified by immunofluorescence using a GM130 antibody and visualized with a Nikon T800 fluorescent microscope. Original magnification ×40.

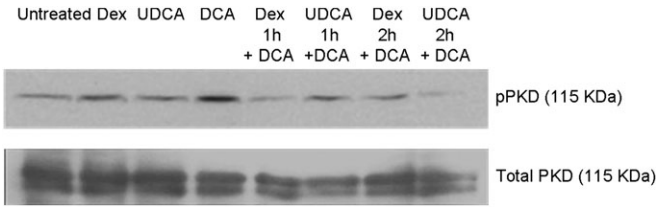

Fig. 4. UDCA and DEX inhibit DCA-induced PKD phosphorylation in HCT116 cells. HCT116 cells were cultured in 0.5% serum for 24 h prior to stimulation. HCT116 cells were treated with 10 nM DEX, 300 μM UDCA or 300 μM DCA for 2 h or pretreated with DEX (10 nM) or UDCA (300 μM) for 1 or 2 h prior to treatment with DCA (300 μM) for 2 h. Cell lysates were prepared and pPKD or total PKD expression was assayed by western blot using an antibody specific to phospho-PKD (Ser 744/748) or total PKD. Blot is representative of three independent experiments.

UC-associated cancer and (v) sporadic colon cancer (Figure 6A). Arbitrary units were used to describe Golgi fragmentation in tissues (Figure 6B). All UC and cancer tissues (both inflammatory and tumour samples) showed significant increases in Golgi fragmentation ($P < 0.005$). There was no significant difference observed between UC tissue without dysplasia, UC tissue with dysplasia or cancer.

Discussion

In this study, we have demonstrated that the bile acid, DCA, can induce Golgi fragmentation in colonic cells by activation of the membrane fission process. This observed Golgi fragmentation is a direct consequence of the activation of PKCη and phosphorylation of PKD. Our finding is consistent with the model proposed by Diaz Anel and Malhotra (33) in which the marine product ilimaquinone induced Golgi fragmentation via hyperactivation of PKD resulting in transport carriers being continuously formed from the TGN until it was reduced to small vesicles. Here, we describe a novel effect of bile acids on Golgi morphology where DCA induced Golgi fragmentation *in vitro* in the HCT116 colon carcinoma cell line. Constitutive Golgi fragmentation has previously been observed in other colonic cancer cell lines, including the SW480, Caco-2, HT-29 and T-84 colon cancer cell lines (25).

In our cell model system, this DCA-induced Golgi fragmentation is mediated by the Golgi-associated PKCη isoform. Localization of this enzyme to the Golgi and TGN appears to play a critical role in regulation of intracellular transport mechanisms. PKCη also has the capacity to translocate to the nucleus in response to signalling through tumour promoters and in this location may play a role in the nuclear machinery for cell proliferation through its interaction with, for example, cyclinE–cdk2 complex (15). Rather than translocation to the nucleus, PKCη remains localized to the Golgi in response to DCA but this change in cellular distribution could also affect cell proliferation and differentiation.

Consistent with previous studies, which have demonstrated that UDCA can antagonize the adverse biological effects of hydrophobic bile acids such as DCA, we have shown here that UDCA could prevent DCA-mediated Golgi fragmentation in colon cancer cells. At a molecular level, UDCA mediates some of its anti-inflammatory effects via activation of the GR (31,34,35) and this is consistent with

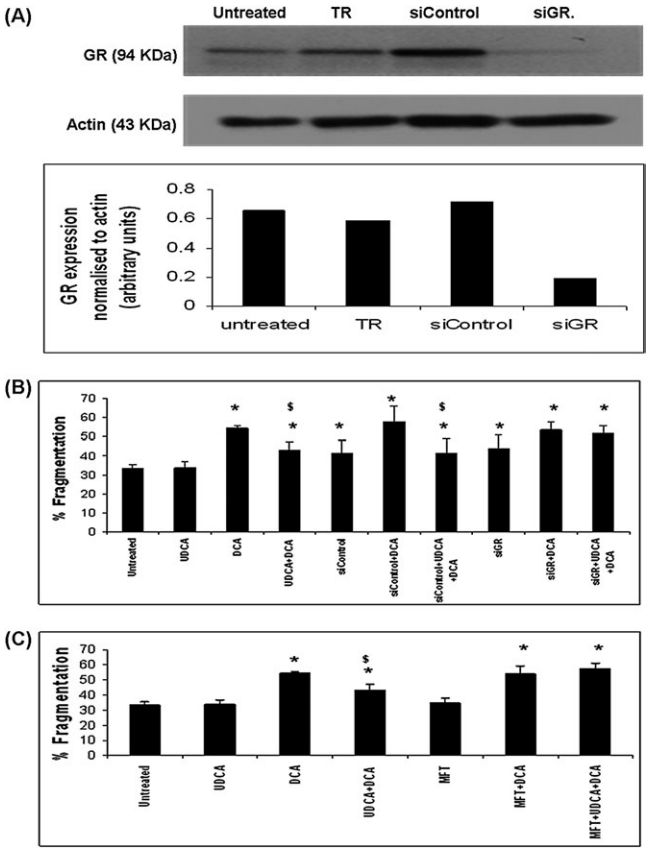

Fig. 5. UDCA-mediated inhibition of DCA-induced Golgi fragmentation was reversed by inhibiting expression of the GR (siGR) or by the glucocorticoid antagonist, MFT. (**A**) GR was knocked down in HCT116 cells using siRNA. A graph of densitometry is included. (**B**) HCT116 cells were treated with 300 μM UDCA (6 h), 300 μM DCA (6 h) or 300 μM UDCA (18 h) + 300 μM DCA (6 h) or transfected with 100 nM siRNA targeting the GR (siGR, 24 h) prior to treatment with UDCA (18 h) and DCA (6 h). Scrambled siRNA sequences (siControl) were used to control for non-specific effects due to siRNA transfection and treated as above with UDCA and DCA. (**C**) The GR was also blocked using 100 nM of the antagonist MFT for 2 h and treated as above. Golgi fragmentation was assessed by high content analysis using the Incell-1000 and Investigator software package. Data were expressed as the mean ± SEM and analysed by analysis of variance with least significant difference post hoc correction ($^{*}P < 0.05$ versus untreated and $^{\$}P < 0.05$ versus DCA treated).

our prior observations that UDCA inhibits cytokine-induced NF-κB induction (5). UDCA-mediated GR activation is ligand independent but facilitates GR translocation to the nucleus through dissociation of the GR–Heat shock protein 90 complex (35). It has previously been demonstrated that UDCA acts on a distinct region of the ligand-binding domain when compared with the classical GR agonist DEX and thus can mediate differential regulation of gene expression by the GR (31). In the current study, UDCA could no longer prevent DCA-induced Golgi fragmentation when the GR was blocked with the GR antagonist MFT or when siRNA was used to knockdown GR expression, indicating that UDCA mediates its effects on Golgi fragmentation via the GR in HCT116 colon cancer cells.

UDCA is currently used clinically for the treatment of liver diseases, such as primary biliary cirrhosis and PSC. Patients with UC and PSC are at an increased risk of developing colon cancer (36). DCA levels are elevated in the colon of patients with PSC, whereas UC patients with neoplasia have increased faecal concentrations of DCA compared with patients without neoplasia (37). UDCA has been shown to act as a chemopreventative agent decreasing the risk for developing colon cancer in these patients (36). In addition, dietary supplementation with UDCA has been shown to inhibit tumour incidence by >50% in response to cholic acid in the azoxymethane colon cancer model (38). The mechanism whereby UDCA improves liver function and DCA-induced damage is unknown. Suggested mechanisms include (i) its ability to create a more hydrophilic bile acid pool counteracting cell damage induced by a hydrophobic bile acid pool; (ii) reduction of inflammation around bile ducts; (iii) improving secretory capacity of hepatocytes in choleostasis and (iv) up-regulation of anti-apoptotic survival signalling pathways (39). We suggest a novel mechanism whereby UDCA inhibits DCA-induced Golgi fragmentation and thus the alterations in intracellular trafficking/signalling associated with this process.

The Golgi apparatus is the organelle responsible for transport, sorting and processing of proteins and lipids from the endoplasmic reticulum. The *cis*-Golgi networks, medial-Golgi networks and TGNs are rich in distinct glycosyltransferase enzymes, which modify O- and N-linked carbohydrate chains on glycoproteins and glycolipids. Disruption of Golgi architecture could therefore result in mis-localization of glycosyltransferases and dissociation from their cognate ligands resulting in alterations in normal protein/lipid processing. Terminal oligosaccharide units expressed on such proteins/lipids are highly specific in their recognition capabilities and are involved in a range of normal cell processes, such as intracellular protein sorting, cell signalling and cell–cell or cell–extracellular matrix adhesion. Alterations in the terminal oligosaccharide units can potentially alter differentiation, proliferation and promote neoplastic progression. Abnormal glycosylation of proteins and lipids is a common observation in malignant cells (40–42) with aberrant glycosylation patterns found in tissue from most colon cancer patients (43–45). Glycosylation alterations also occur in UC, Crohn's disease, UC-associated cancer and sporadic colon cancer with increased expression of Thomsen Friedenreich (TF) and sialyl tenascin

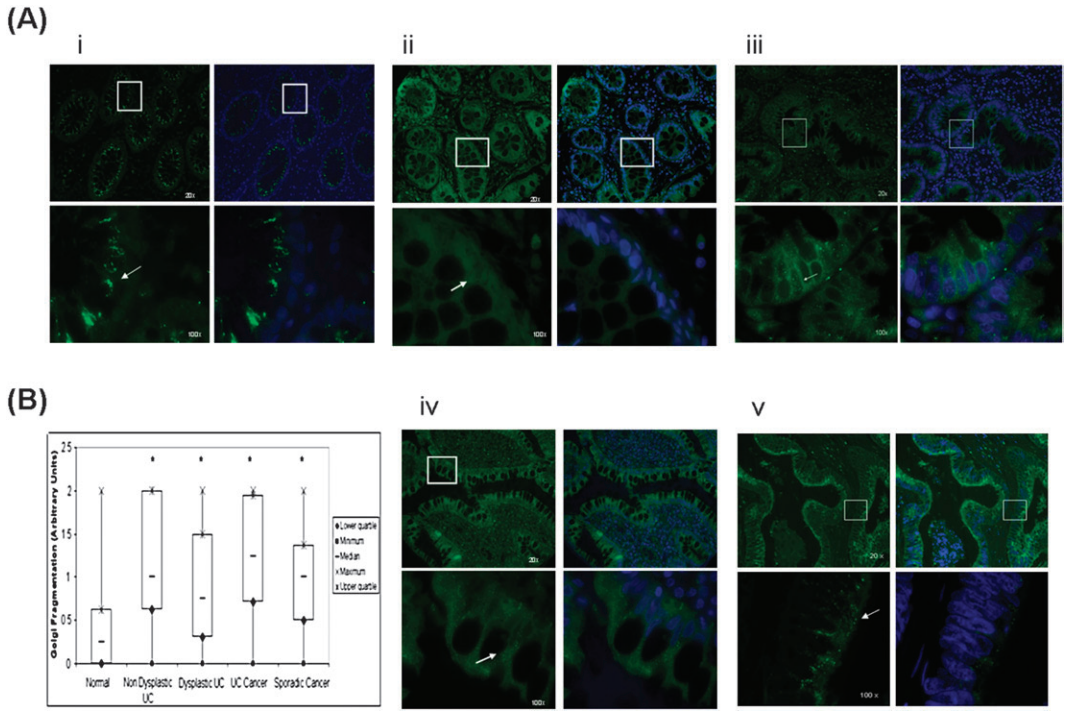

Fig. 6. Golgi fragmentation in patients with UC and colon cancer. (**A**) Golgi structure was examined in five sets of tissue: (i) no history of malignant disease (normal); (ii) UC with no progression to dysplasia (non-dysplastic); (iii) UC with progression to dysplasia (dysplastic); (iv) colorectal cancer on background of UC (UC cancer) and (v) sporadic colon cancer. Golgi (green, as indicated by white arrow) were visualized using a TGN antibody TGN46 and an AlexaFluor fluorescent secondary antibody. (**B**) Biopsies classified as completely fragmented Golgi were assigned arbitrary scores of 0 for non-fragmented Golgi, 1 for partially fragmented Golgi and 2 for fully fragmented Golgi. Data are expressed as medians and interquartile ranges; $^{*}P < 0.05$.

oncofoetal antigens that are high-risk markers for cancer progression in inflammatory bowel disease (46,47). These glycosylation changes have been observed in the absence of dysplasia and can occur prior to malignant transformation (44). Altered N-linked glycosylation leading to over-expression of sialyl Lewis A and sialyl Lewis X carbohydrates is found in tumours and correlates with metastasis (48).

Bile acids have previously been shown to activate PKCα and PKCδ in hepatocytes by facilitating enzyme association with phospholipids and increasing cell membrane diacylglycerol (DAG) content. Bile acids do not appear to bind to DAG (49) but instead stimulate its formation in rat hepatocytes (50) possibly through phospholipase C activation (51). Trichloroacetic acid and DCA have also been shown to activate DAG formation in normal colon and colonic tumour cell extracts (52). PKC activation is highly dependent on physiochemical properties of the lipid membrane and PKCη activity is more sustained at the Golgi due to prolonged DAG accumulation at this site (53).

We have observed Golgi fragmentation in biopsies obtained from patients with the inflammatory condition UC and in patients with colon cancer. We did not observe differences in levels of Golgi fragmentation between UC and cancer patients nor did we observe a progression between UC, dysplasia and cancer. It is possible that there are multiple processes that could lead to Golgi fragmentation in inflammation and cancer. It has been proposed that increased levels of bile acids may play a role in both inflammatory bowel disease and cancer (4). Hence, it is possible that Golgi fragmentation may play a role in the pathogenesis of both UC and cancer through either single or multiple mechanisms.

The effects of UDCA in inhibiting Golgi fragmentation may play a role in several of its pharmacological effects including possible effects in liver disease and chemopreventative effects in patients with premalignant colonic diseases, such as extensive UC. These studies demonstrate a potential link between bile acids and Golgi fragmentation that may be involved in the pathogenesis of both benign and malignant disease of the gastrointestinal tract.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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