

Plasma membrane calcium ATPase 4 and the remodeling of calcium homeostasis in human colon cancer cells

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A remodeling of calcium homeostasis has been identified as a characterizing feature of some cancers. Possible consequences of this include alterations in many pivotal physiological responses including apoptosis, proliferation and gene transcription. An alteration in calcium homeostasis can occur via changes in the expression of proteins that transport calcium and examples of cancers where this is seen includes the prostate and breast. A specific isoform of the calcium efflux pump, plasma membrane Ca²⁺-ATPase (PMCA) 4, is significantly upregulated during differentiation of the HT-29 colon cancer cell line suggesting that it may also be altered in colon cancer. We now report that differentiated HT-29 colon cancer cells have pronounced plasma membrane PMCA4 localization, consistent with augmented calcium efflux. Assessment of PMCA4 transcription in human colon cancer samples suggests that PMCA4 is significantly ($P < 0.000001$) downregulated early in the progression of some colon cancers as these cells become less differentiated. Inhibition of PMCA4 using small interfering RNA did not induce cell death or augment sensitivity to the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or tumor necrosis factor-related apoptosis-inducing ligand. Reversing the colon cancer remodeling of PMCA4 by overexpression reduced cellular proliferation ($P < 0.01$) and downregulated transcription of the calcium sensitive early response gene *FOS*. Our studies suggest that the remodeling of the calcium signal in colon cancer is associated with compromised calcium efflux at a level that promotes proliferative pathways while avoiding increased sensitivity to apoptotic stimuli.

Introduction

The level of intracellular free calcium is highly regulated and is a key mechanism in controlling a range of cellular processes, many of which are important pathways in tumorigenesis (1,2). Alterations in the transcription of ion channels permeable to calcium and active transporters of calcium such as P-type ATPases occur in a variety of specific cancer types (1). The best characterized are increases in the expression of the calcium permeable ion channels TRPM8 and TRPV6 in prostate cancer (3–5). The modulation of these channels alters proliferation and cell viability of prostate cancer cells lines (6,7). By comparison, there are fewer studies of active transporters of the calcium ion in the context of cancer and the potential consequences of their altered expression (8–12).

The plasma membrane Ca²⁺-ATPase (PMCA) is encoded by four genes and is alternatively spliced to generate over 30 distinct isoforms

Abbreviations: ATP, adenosine triphosphate; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; mRNA, messenger RNA; PCR, polymerase chain reaction; PMCA, plasma membrane Ca²⁺-ATPase; SERCA, sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase; siRNA, small interfering RNA; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

(13) and is an example of a P-type ATPase (14). PMCA4 actively extrude Ca²⁺ ions across the plasma membrane and are responsible for maintaining the large ionized-free Ca²⁺ gradient in resting cells and restoring resting intracellular free Ca²⁺ after cell activation (14,15). Isoform-specific changes in PMCA-mediated efflux occur during the differentiation of colon cancer cell lines, with upregulation of PMCA4 expression during both post-confluence and chemically induced differentiation such as with sodium butyrate (16,17). In contrast, PMCA1 transcription changes are not associated with the differentiation of colon cancer cell lines (16,17). PMCA4-induced changes in expression occur in a variety of colon and gastric cancer cell lines including HT-29 and Caco-2 (17). PMCA4 was believed to be, along with PMCA1, an essential 'housekeeper' (15). However, knockout of the PMCA1 gene is embryonically lethal in mice (18). In contrast, PMCA4-knockout mice are viable, and studies using these animals suggest that PMCA4 may play a role in sperm motility and other specific processes (18). Changes in PMCA4 expression during differentiation may in part explain differences in calcium homeostasis induced by differentiation of colon cancer cells, such as delayed recovery of intracellular free Ca²⁺ after stimulation (10).

The upregulation of PMCA4 expression during the differentiation of colon cancer cells suggests that the less differentiated state may be associated with compromised calcium efflux. However, it is unclear if altered levels of PMCA4 are a characterizing feature of some colon cancers, as it is for calcium pumps of the endoplasmic reticulum (8–11). It is also unclear if induction of differentiation of colon cancer cells results in targeting of PMCA4 to the plasma membrane where it is required to alter calcium efflux and hence regulate cellular signaling.

The potential significance of altered expression of PMCA is complex. This complexity arises from the role of intracellular free Ca²⁺ in seemingly opposing processes, such as apoptosis and proliferation (1,14). This makes the consequences of a remodeling of calcium efflux in colon cancer cells difficult to predict. Compromised PMCA-mediated calcium efflux may promote sensitivity to apoptosis inducers or could alter the transcription of cancer-associated genes important in proliferation (1,2). For example, caspase-mediated inhibition of PMCA-mediated Ca²⁺ efflux promotes calcium overload, a key trigger of apoptosis (19), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis is enhanced by pharmacological inhibitors of the mitochondrial Na⁺/Ca²⁺ exchanger and the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCA) (20). Conversely, studies looking at the overexpression of CACNA1E, a calcium influx channel subunit overexpressed in some Wilms' tumors, suggest that a net calcium gain across the plasma membrane (which could also occur with compromised PMCA-mediated calcium efflux) is associated with the upregulation of early response genes including *FOS*, which suggests increased cell proliferation (21). The potential role of a remodeling of calcium efflux in colon cancer cells in this context has not been assessed.

In this study, we describe the localization of PMCA4 during colon cancer cell line differentiation and the level of PMCA4 in colon cancers. We also assessed the consequences of small interfering RNA (siRNA)-mediated PMCA4 knockdown on the sensitivity of HT-29 colon cancer cells to apoptosis inducers and calcium homeostasis, as well as the consequences of an induced remodeling of PMCA-mediated efflux through overexpression of PMCA4 in HT-29 colon cancer cells. These studies suggest that remodeling of calcium signaling (2) occurs in some colon cancers through compromised calcium efflux via alterations in PMCA expression, at a level that may bestow a growth advantage while avoiding reduced cellular viability and increased sensitivity to apoptotic stimuli.

Materials and methods

Cell culture and human clinical samples

HT-29 human colon cancer cells were cultured in normal growth RPMI-1640 media (Invitrogen, Mount Waverley, VIC, Australia), supplemented with 10% fetal bovine serum (Sigma Aldrich, Castle Hill, NSW, Australia), 2 mM L-glutamine (Invitrogen), penicillin G (100 U/ml; Invitrogen) and streptomycin sulfate (100 µg/ml; Invitrogen) and incubated at 37°C in a humidified 5% CO₂/95% air incubator as described previously (16). Human Colon-Matched cDNA Pair Panel samples (Clontech, Mountain View, CA) are isolated from human colon tumors and the corresponding surrounding normal tissue from individual patients. Samples are invasive colon adenocarcinomas from two male (A and B) and three female (B, D and E) patients, with metastases to lymph nodes reported for patients B and D.

Immunofluorescence analysis

HT-29 cells were seeded at 12×10^4 cells on glass cover slips placed in each well of a 24-well plate and cultured in normal growth media with the media renewed every 2 days until confluence or the required time post-confluence was attained. The cells were fixed in 4% paraformaldehyde (ProSciTech, Kirwan, QLD, Australia) followed by ice-cold methanol and non-specific binding sites were blocked with 5% goat serum (VectorLabs, Burlingame, CA) and 1% bovine serum albumin (Sigma Aldrich) in buffer (phosphate-buffered solution containing 0.1 mM CaCl₂ and 1 mM MgCl₂, pH 7.4). The primary antibody, goat anti-mouse PMCA4 antibody (JA9; Affinity BioReagents, Golden, CO), and fluorescent secondary antibody, goat anti-mouse Hilyte Fluor™ 488-labeled antibody (AnaSpec, Fremont, CA), were both used at a dilution of 1:100. Cell nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (200 ng/µl; Sigma Aldrich). Cover slips were mounted on the slides using fluorescent mounting medium (Dako, Glostrup, Denmark) and PMCA4 localization was observed using a Nikon Eclipse TE 300 inverted fluorescent microscope based on the following excitation and emission wavelengths: 488 and 550 nm for PMCA4 and 380 and 520 nm for 4',6-diamidino-2-phenylindole dihydrochloride staining, respectively. Two independent experiments were performed for each time point and for each experiment, two cover slips were stained at each time point. Images were taken from each cover slip and image acquisition used MetaFluor® Version 4.01 imaging software (Universal Imaging Corporation, Molecular Devices, Downingtown, PA).

Real-time reverse transcription-polymerase chain reaction

Total RNA was isolated using the RNeasy Plus Mini kit (Qiagen, Doncaster, VIC, Australia) following the manufacturer's protocol. Real-time reverse transcription-polymerase chain reaction (PCR) was used to quantitate target gene expression using a 7500 Real-Time PCR System (Applied Biosystems). PMCA1, PMCA4 and ALPI were amplified as described previously (16,22–24) and using the PMCA1 Taqman Gene Expression assay Hs00155949_m1. *FOS* was amplified using the Taqman Gene Expression assay Hs00170630_m1 and the Taqman Universal PCR Master Mix (Applied Biosystems) after reverse transcription using the Omniscript Reverse Transcriptase (Qiagen). All amplifications used universal cycling conditions (Applied Biosystems). Target messenger RNA (mRNA) levels were normalized to the endogenous control 18S ribosomal RNA (Applied Biosystems) and data were analyzed using the threshold cycle values (Ct) to calculate ΔCt , which was then expressed as relative to the appropriate control (see figure legends) to determine $\Delta\Delta Ct$. Fold change was calculated from ΔCt with the formula $2^{-\Delta\Delta Ct}$ as described previously (25).

Microarray data

Normal colon and colon tumor microarray data were generated by Genelogic (Gaithersburg, MD) using the Affimetrix HGU133P chip with probe 205410_s_at. Signal intensity values from Microarray Analysis Suite version 5 were utilized with a scaling factor of 500 for all analysis. Sample numbers included in the analysis were: normal colon from patients without colon cancer, 79; normal colon from patients with colon cancer, 122; colon adenocarcinoma, 85 and benign colon tumor, 27. *P* values between tumor and normal samples were calculated using Student's *t*-test with the unpaired, two tails and unequal variants condition.

PMCA4 siRNA

PMCA4 expression was inhibited using Dharmacon ON-TARGETplus SMARTpool PMCA4 siRNA (ATP2B4; catalog number 006118) and the controls ON-TARGETplus Non-Targeting siRNAs and ON-TARGETplus Non-Targeting Pool siRNA. Dharmacon ON-TARGETplus SMARTpool PMCA4 siRNAs are a pool of four specific siRNA sequences specific for PMCA4. The siRNAs have dual-strand modification (26) and use an algorithm to minimize seed region matches (27), both designed to reduce off-target effects.

HT-29 cells were seeded at a density of 1×10^4 cells per well into 96-well plates in normal growth media without antibiotics. siRNA transfection was according to the manufacturer's instructions using the transfection agent DharmaFECT 4 and a final total siRNA concentration of 100 nM (i.e. 25 nM/siRNA) in each well. Cells were incubated for 24 h prior to RNA isolation and 72 h for protein isolation and inhibition of PMCA4 expression determined via reverse transcription-PCR and immunoblotting for RNA and protein expression, respectively. In addition to non-targeting siRNA, controls included mock-transfected cells (transfection agent only and siRNA volume replaced with $1 \times$ siRNA buffer) and non-transfected cells.

Immunoblotting

A description of the protein isolation and immunoblotting for PMCA4 and β -actin can be found in Aung *et al.* (16). Lysed cells were pooled from four different wells of the 96-well plates to ensure adequate protein for immunoblotting. For each treatment condition, two different pooled samples were prepared from eight wells. Pooled protein lysates (20 µl) were loaded into each well and separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel before being transferred onto a nitrocellulose membrane. The membrane was probed for PMCA4 expression using the PMCA4-specific primary monoclonal goat anti-mouse antibody JA9 (1:1000 dilution; Affinity Bioscience) and anti-mouse β -actin antibody (1:10 000 dilution; Sigma Aldrich) that was used as an internal loading control. The secondary antibody, goat anti-mouse IgG horseradish peroxidase conjugated, was used at a dilution of 1:2000 (Bio-Rad, Gladesville, NSW, Australia). Antibody protein complexes were visualized using ECL™ Western Blotting analysis system (GE Health Care, Richmond, VIC, Australia) in accordance with the manufacturer's protocol.

Assessment of cell viability after TRAIL and/or carbonyl cyanide 3-chlorophenylhydrazone treatment

Experiments consisted of either untransfected HT-29 cells or HT-29 cells that had been transfected with siRNA (PMCA4 siRNA or non-targeting pool siRNA). Forty-eight hours after transfection, carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma Aldrich, 20 µM final concentration in the well) was added to the appropriate wells. CCCP was dissolved in dimethyl sulfoxide and the final concentration of dimethyl sulfoxide in each well was never >0.5%. After 1 h, media with TRAIL dissolved in water (0.5 mg/ml) was then added to the appropriate wells to obtain a final TRAIL concentration of 50 ng/ml per well and the cells incubated for a further 23 h before the assessment of cell viability. Viable cell number was determined using the CellTiter 96® Aqueous One Solution reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, Promega, Alexandria, NSW, Australia] following the manufacturer's protocol. Briefly, treatment media was replaced with normal RPMI-1640 media and CellTiter 96® Aqueous One Solution reagent (20 µl) was added to each well of the 96-well plate and incubated for 2 h at 37°C followed by measuring the absorbance at 490 nm using a Bio-Rad Model 550 microplate reader.

Measurement of intracellular Ca²⁺

The intracellular cytosolic-free Ca²⁺ concentration was measured after the addition of adenosine triphosphate (ATP) or neurotensin using the fluorescence imaging plate reader FLIPR^{TETRA} (Molecular Devices, Downingtown, PA) and the Ca²⁺-sensitive dye Fluo-4 AM (Molecular Probes, Invitrogen). Briefly, HT-29 cells were seeded in 96-well CellBIND plates (Corning) at a density of 1×10^4 cells per well in antibiotic-free RPMI-1640 growth media for 24 h before transfection with siRNA (PMCA4 siRNA or non-targeting pool siRNA) as described above. After 72 h, the transfection medium was removed and cells were loaded with Fluo-4 AM (4 µM) for 30 min at 37°C. The cells were then washed and incubated for 15 min at room temperature prior to experiments. Relative [Ca²⁺]_i was determined in the presence of approximate maximal and submaximal concentrations of ATP (1 mM and 22.1 µM) and neurotensin (1 µM and 12.8 nM), determined from dose response curves (data not shown). Response over baseline was used to compare relative [Ca²⁺]_i and fluorescence was determined at 470–495 nm excitation and 515–575 nm emission over an 800 s period. Results are given as the mean \pm SEM (*n* = 11–14) of three or four different wells from three independent experiments. Wells were excluded prior to data analysis based on poor or uneven dye loading (four wells in total).

Generation of PMCA4 overexpressing stable HT-29 cells and assessment of proliferation

The human PMCA4b expression vector [pSG5-PMCA4b; (28)] was a gift from Professor Ernesto Carafoli and Dr Marisa Brini (University of Padova, Italy) and Associate Professor Melissa Brown (University of Queensland, Australia) kindly provided the pSG5 plasmid. HT-29 cells were transfected with either pSG5-PMCA4b or empty vector pSG5 and the hygromycin-resistant gene encoding plasmid pTK-Hyg (Clontech) using Lipofectamine 2000

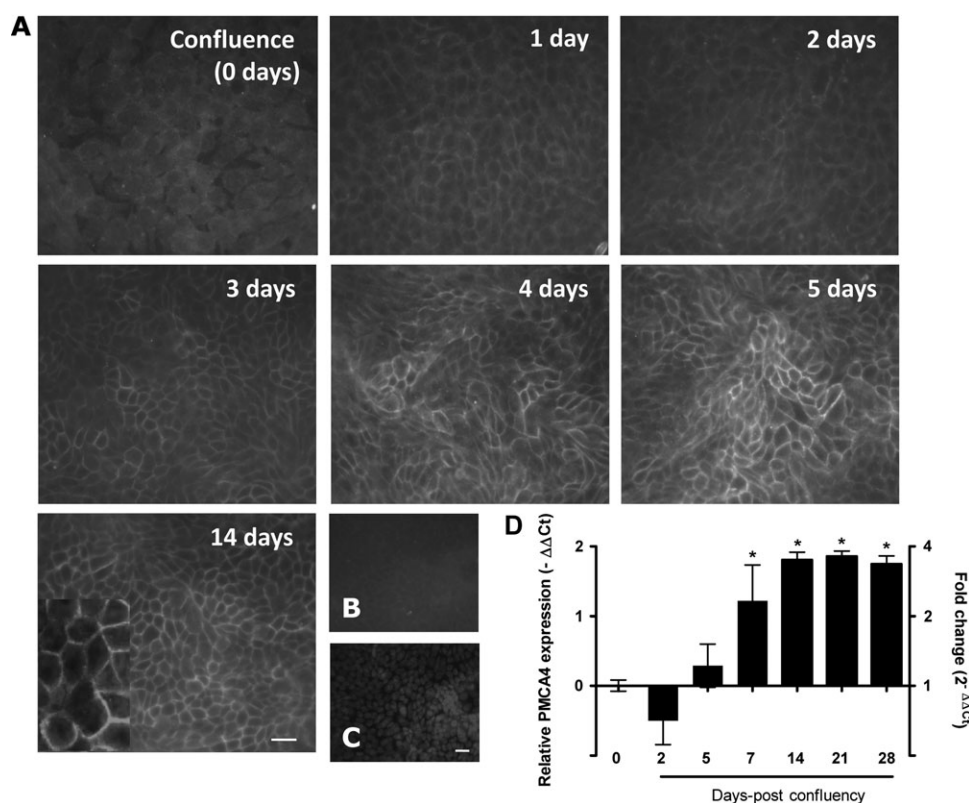


Fig. 1. PMCA4 localization and relative PMCA4 mRNA levels during post-confluence mediated differentiation of HT-29 colon cancer cells. Cells were grown on glass cover slips in 24-well plates until confluence and post-confluence was attained. Cells were probed with antibody to PMCA4 and detection was via a fluorescent secondary antibody. mRNA was also isolated from the cells at confluence and post-confluence and PMCA4 levels quantitated using real-time reverse transcription-PCR, normalized to 18S ribosomal RNA and expressed relative to PMCA4 mRNA levels at confluence (day 0). (A) PMCA4 localization in confluent cultures (0 days) to 14 days post-confluence, scale bar = 20 μ m. Each panel is representative of 16 fields from 4 cover slips from 2 independent passages. Insert panel for 14 days post-confluence shows magnified view of a 4',6-diamidino-2-phenylindole dihydrochloride (blue) and PMCA4 (green) overlay at day 14. (B) Staining in the absence of anti-PMCA4 JA9 antibody in day 14 cultures. (C) Corresponding 4',6-diamidino-2-phenylindole dihydrochloride nuclear staining from day 14 cultures, scale bar = 20 μ m. (D) Relative PMCA4 mRNA levels during days post-confluence-induced differentiation assessed using real-time reverse transcription-PCR, * $P < 0.05$ (analysis of variance) compared with day 0 (confluence).

(Invitrogen). Stable colonies were created by selection with hygromycin. Real-time reverse transcription-PCR was used to confirm PMCA4 overexpression. The passage number of stably transfected cells was between 5 and 15 for all experiments. The proliferation rate of the stable cell lines and the parental HT-29 cell line was assessed by seeding the cells at a density of 5×10^3 cells into a 96-well plate. Growth rate was assessed at 24, 48, 72, 96 and 120 h using the CellTiter 96® Aqueous One Solution reagent as described above.

Statistical analysis

Data analysis was performed using GraphPad Prism version 4.0 for Windows (GraphPad Software) and significant differences were analyzed using either a one-way analysis of variance with a *post hoc* Tukey's test or Dunnett's test or Student's *t*-test for unpaired samples. Significance was considered at $P < 0.05$ and is described in individual figure legends.

Results

PMCA4 and HT-29 colon cancer cell line differentiation

The reported increased expression of PMCA4 during colon cancer cell line differentiation (16,17) will only alter calcium signaling if PMCA4 is targeted to the plasma membrane to efflux Ca^{2+} ions at rest and after stimuli. PMCA4 plasma membrane localization was evident even before the maximum induction in PMCA4 transcription (Figure 1A and D). These studies also indicate that in non-differentiated HT-29 cells, PMCA4 levels are not only lower (Figure 1D) but also localization of PMCA4 to the plasma membrane is less pronounced (Figure 1A), which would further compromise PMCA-mediated Ca^{2+} efflux.

Table 1. PMCA4 and PMCA1 mRNA expression in colon tumors relative to normal matched surrounding tissue

Patient	PMCA4 mRNA level relative to matched sample	PMCA1 mRNA level relative to matched sample
A	0.43	0.18
B	0.94	1.32
C	0.84	1.14
D	0.17	0.11
E	0.48	0.34

PMCA mRNA levels were quantitated using real-time reverse transcription-PCR and normalized to the level of 18S ribosomal RNA in that sample. Results are expressed as relative to the normal matched surrounding tissue.

PMCA4 is reduced in some human colon cancers

Genes that are often upregulated during differentiation in colon cancer cell lines are conversely downregulated in many colon cancers, reflective of the less differentiated phenotype of the tumor. To assess whether reduced transcription of calcium efflux pumps may be a feature of some colon cancers, we assessed PMCA4 levels in a small bank of human colon cancers and matched surrounding normal tissue (Table 1). Reduced PMCA4 mRNA levels were associated with tumors from some patients, with tumor/matched tissue ratios as low as 0.17. PMCA4 levels were not elevated in any of the tumors compared

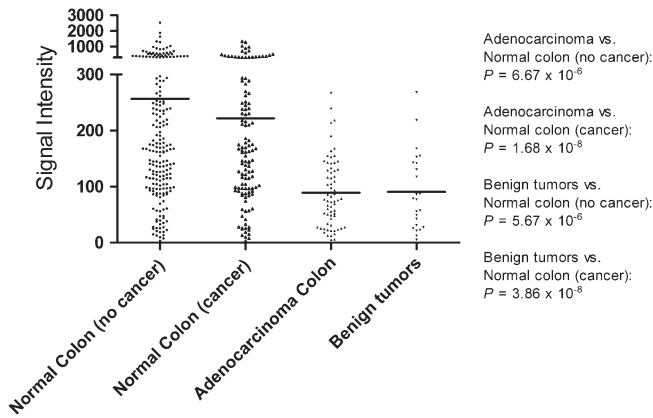


Fig. 2. RNA isolated from normal colons from patients with ($n = 122$) and without ($n = 79$) cancers, as well as colon adenocarcinomas ($n = 85$) and benign colon tumors ($n = 27$) were hybridized to the affymetrix chip HGU133P. Normalized microarray signal intensities of PMCA4 were plotted against each tissue category. P values of tumor versus normal colon comparison were calculated using Student's t -test and are listed on the right side of the figure.

with matched surrounding tissue. PMCA1 levels were reduced in three of five human colon cancers, two had elevated levels of PMCA1 (Table I). We chose PMCA4 for further study because of its association with the differentiation of HT-29 cells and its downregulation in the small bank of human colon cancers. The initial results in the colon tumors prompted us to examine PMCA4 levels using complementary DNA microarray data and compare normal colon tissue isolated from cancer and non-cancer patients with colon adenocarcinomas and benign tumors from a larger patient population. Mean PMCA4 levels were significantly reduced in both colon adenocarcinomas and benign tumors compared with normal colon isolated from either cancer or non-cancer patients (Figure 2).

Inhibition of PMCA4 in HT-29 cells

Due to the link between calcium overload and cell death (29), we assessed the potential consequence of selective PMCA4 inhibition on the viability of HT-29 colon cancer cells. PMCA4 siRNA significantly reduced the level of PMCA4 mRNA (Figure 3A) with no effect on the related isoform, PMCA1 (Figure 3B), which is also expressed in HT-29 cells (16) and would contribute to calcium efflux from the cell. PMCA4 siRNA also exhibited pronounced inhibition of PMCA4 protein (Figure 3C).

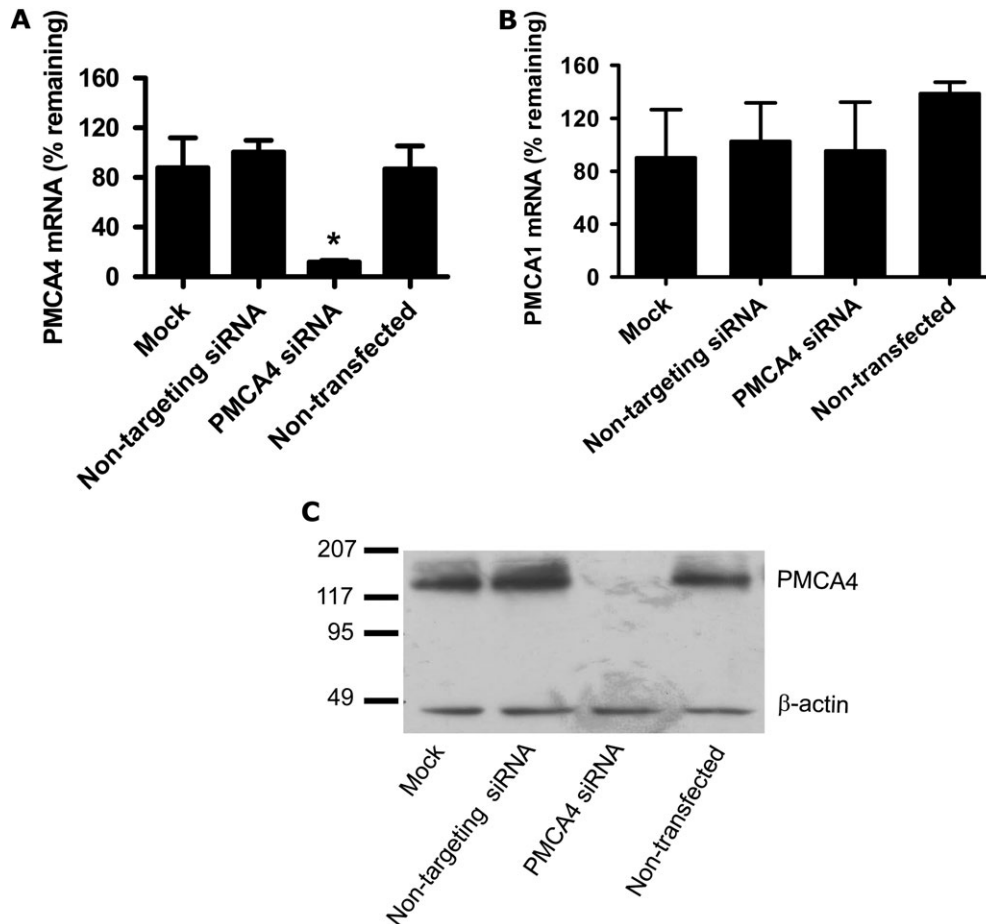


Fig. 3. siRNA-mediated knockdown of PMCA4 in HT-29 colon cancer cells. Cells were grown in 96-well plates and PMCA4 expression was inhibited using Dharmacon ON-TARGET^{plus} SMARTpool siRNA. RNA and protein were isolated 24 h (RNA) and 72 h (protein) after transfection. Levels were quantitated using either real-time reverse transcription-PCR or immunoblotting. (A) PMCA4 mRNA levels in HT-29 cells treated with or without PMCA4 siRNA for 24 h. Controls included mock-transfected cells (transfection agent only), ON-TARGET^{plus} Non-Targeting siRNA and non-transfected cells. Each bar represents the mean \pm SD ($n = 3$ individual wells); * $P < 0.05$ (analysis of variance) compared with non-targeting siRNA. (B) PMCA1 mRNA levels in HT-29 cells treated with or without PMCA4 siRNA for 24 h. Controls included mock-transfected cells (transfection agent only), ON-TARGET^{plus} Non-Targeting siRNA and non-transfected cells. Each bar represents the mean \pm SD ($n = 3$ individual wells). (C) Immunoblot for PMCA4 protein, using the JA9 monoclonal antibody, in HT-29 cells treated with or without PMCA4 siRNA for 72 h. Controls included mock-transfected cells (transfection agent only), ON-TARGET^{plus} Non-Targeting siRNA and non-transfected cells. β -Actin served as a loading control. Immunoblot depicted is representative of two blots from separate pooled protein isolates.

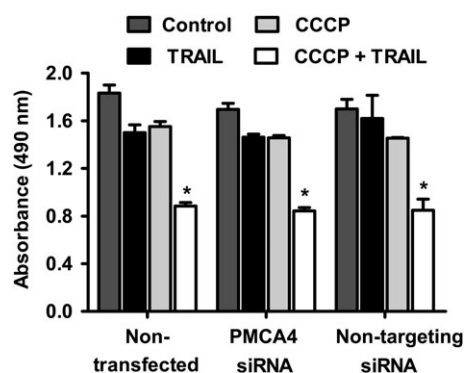


Fig. 4. Consequences of PMCA4 knockdown on cellular viability of HT-29 cells in response to TRAIL and/or CCCP. Non-transfected or siRNA-transfected HT-29 cells (PMCA4 or non-targeting pool) were treated with or without CCCP (20 μ M) 48 h after siRNA treatment and then after 1 h exposure to CCCP, cells were incubated in media with or without TRAIL (50 ng/ml) for a further 23 h. Cellular viability was then measured using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Each bar represents the mean \pm SEM of individual wells ($n = 4$) and this figure is representative of two independent experiments from different cell passages. A significant difference ($P < 0.05$, analysis of variance) was seen between the presence of CCCP and TRAIL compared with the appropriate control for each transfection group. No significant difference was seen between transfection groups for each treatment.

Effect of PMCA4 inhibition on HT-29 cell viability and sensitivity to TRAIL

Consistent with previous studies (30), HT-29 colon cancer cells were resistant to TRAIL-mediated cell death, although this was overcome by concurrent treatment with CCCP (Figure 4). Both CCCP and TRAIL alone exhibited only modest effects on HT-29 cell viability; however, in combination they exhibited a pronounced and significant effect (Figure 4). Inhibition of the endoplasmic reticulum calcium pump, SERCA, can promote TRAIL sensitivity in some tumor cells (31,32). Given this, we assessed if inhibition of PMCA4 also promoted the effects of TRAIL and/or CCCP in HT-29 cells. However, we saw no effect of PMCA4 inhibition on either HT-29 cell viability *per se* or sensitivity to the effects of CCCP and/or TRAIL (Figure 4).

Effect of PMCA4 siRNA on agonist-induced increases in $[Ca^{2+}]_i$

The expression of PMCA1 in HT-29 cells could mask the consequences of siRNA-mediated inhibition of PMCA4. To assess this directly, we compared agonist-induced increases in $[Ca^{2+}]_i$ in the absence and presence of PMCA4 inhibition. Maximal and submaximal concentrations of ATP and neurotensin were determined (data not shown) and used to compare responses in HT-29 cells. ATP-induced $[Ca^{2+}]_i$ transients in the HT-29 cells were associated with a more pronounced calcium influx phase than that seen with neurotensin (Figure 5A and B). PMCA4 inhibition increased peak $[Ca^{2+}]_i$ after maximal concentrations of both ATP and neurotensin (Figure 5A–C), as well as in the presence of submaximal concentrations of ATP (Figure 5C), despite the maintained transcription of PMCA1.

Reverse remodeling of reduced PMCA4 expression in HT-29 colon cancer cells

Given the reduced level of PMCA4 in some colon cancers (Table I and Figure 2) and our results showing that in a colon cancer cell line, when PMCA4 is inhibited there is an augmentation of $[Ca^{2+}]_i$ responses after stimulation (Figure 5), we wanted to assess the consequences for the cancer cell if the reduction in PMCA4 was reversed. To achieve this 'reverse remodeling', we overexpressed PMCA4 in HT-29 cells in the absence of differentiating agents. Stable overex-

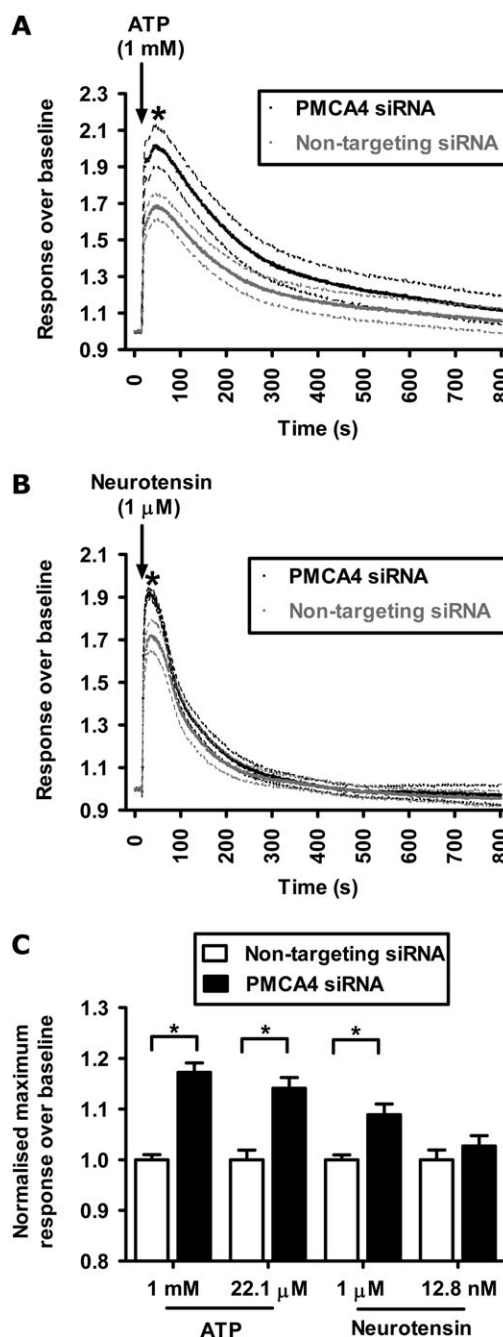


Fig. 5. Effect of PMCA4 siRNA-mediated knockdown on $[Ca^{2+}]_i$ in HT-29 cell lines. Cells were transfected in 96-well plates with either PMCA4 siRNA or non-targeting pool siRNA for 72 h and then loaded with the Ca^{2+} probe (Fluo-4 AM) and imaged using the FLIPR^{TETRA}. (A) $[Ca^{2+}]_i$ during stimulation with ATP 1 mM. (B) $[Ca^{2+}]_i$ during stimulation with 1 μ M neurotensin. For (A and B) each trace represents the mean \pm SD ($n = 4$ wells) and are representative of three independent experiments from different cell passages; * $P < 0.05$ (Student's *t*-test) denotes the statistical significance of the mean maximum response over the baseline as compared with the non-targeting siRNA-transfected control. (C) Each bar represents the response to either ATP or neurotensin as labeled. Responses were normalized to non-targeting siRNA control for each agonist and each bar represents the mean \pm SEM ($n = 11$ –14) from three independent experiments from different cell passages; * $P < 0.05$ (Student's *t*-test).

pression of PMCA4 (Figure 6A) did not induce the transcription of the differentiation marker ALPI (Figure 6B) or produce compensatory changes in PMCA1 transcription (Figure 6C); however, there was

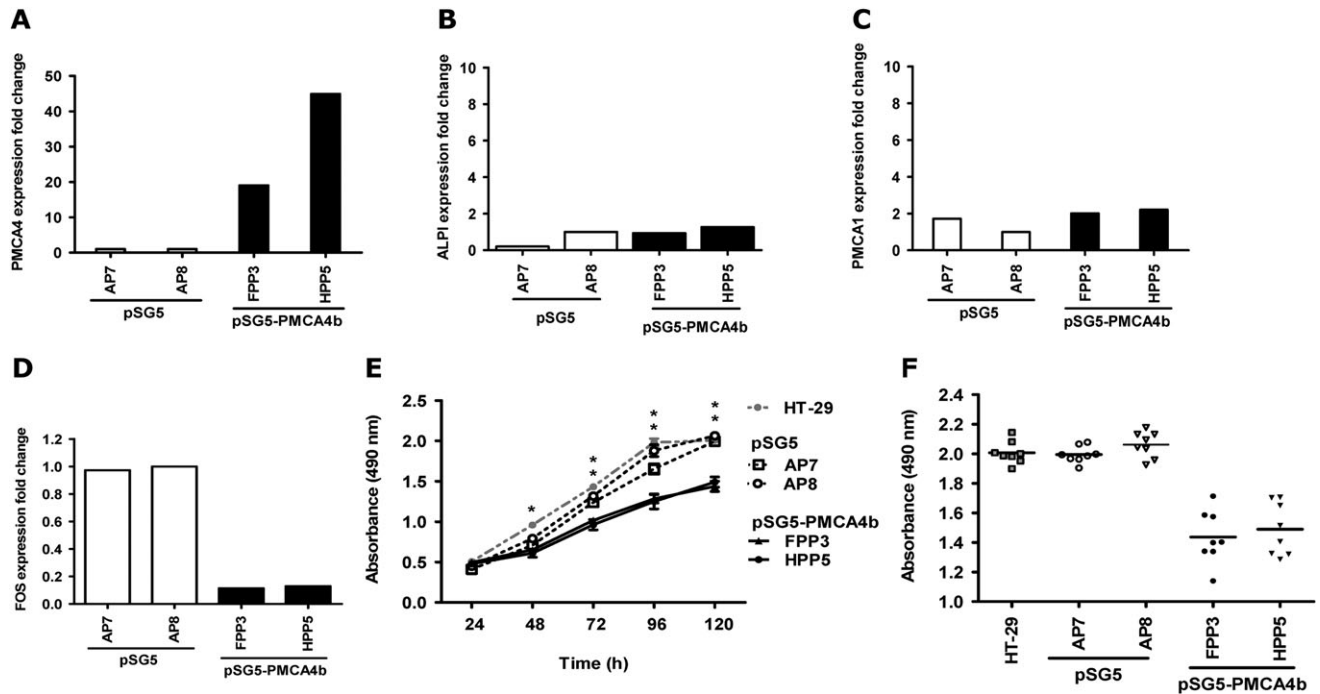


Fig. 6. Consequences of reversing the remodeling of PMCA4 expression in HT-29 colon cancer cells. (A) Relative PMCA4 mRNA in control (AP7 and AP8) and PMCA4 overexpressing (FPP3 and HPP5) HT-29 colon cancer cells. (B) Relative ALPI mRNA levels in control (AP7 and AP8) and PMCA4 overexpressing (FPP3 and HPP5) HT-29 colon cancer cells. (C) Relative PMCA1 mRNA levels in control (AP7 and AP8) and PMCA4 overexpressing (FPP3 and HPP5) HT-29 colon cancer cells. (D) Relative *FOS* mRNA levels in control (AP7 and AP8) and PMCA4 overexpressing (FPP3 and HPP5) HT-29 colon cancer cells. For (A–C) real-time reverse transcription–PCR was used to quantitate mRNA levels and results are presented as fold change in expression level relative to AP8 cell line and normalized to 18S ribosomal RNA. Results shown are the mean ($n = 3$). (E) Proliferation of parental HT-29 cells, control (AP7 and AP8) and PMCA4 overexpressing (FPP3 and HPP5) HT-29 cells ($n = 8$, mean \pm SEM) from two independent passages. The symbol indicates *** AP8 is significantly ($P < 0.01$) different to HPP5 and HT-29, *** indicates AP8 is significantly ($P < 0.01$) different to FPP3 and HPP5. (F) Relative cell numbers 120 h after plating in individual microplate wells of parental HT-29 cells, control (AP7 and AP8) and PMCA4 overexpressing (FPP3 and HPP5) HT-29 cells ($n = 8$) from two independent passages.

a reduction in the expression of the early response gene *FOS* (Figure 6D). Given that the pro-proliferative effects of CACNA1E overexpression (a calcium influx channel subunit overexpressed in some Wilms' tumors) is linked to the altered expression of early response genes (21), we also assessed the ability of PMCA4 overexpression to reduce the proliferation of HT-29 colon cancer cells. Reversing the remodeling of calcium efflux through PMCA4 overexpression was associated with significantly ($P < 0.01$) reduced proliferation rates (Figure 6E and F).

Discussion

Alterations in the levels of calcium transporters are an increasingly recognized feature of some cancers (1). However, the consequences of such alterations are difficult to predict due to the diverse processes regulated by the Ca^{2+} signal (1,2) and because of potential compensatory mechanisms via the activity or expression of other calcium transporters (1). We observed that PMCA4 expression was downregulated in some colon tumors and that globally PMCA4 expression was significantly less in adenocarcinomas compared with normal colon samples ($P < 10^{-6}$). To realize the significance of a downregulation in PMCA4, in this study, we examined the role of PMCA4 in the context of differentiation and proliferative pathways. The results collectively indicate that the remodeling of calcium efflux occurs at a level that provides colon tumors with a growth advantage, but does not promote cell death or sensitivity to apoptotic inducers.

Differentiation of colon cancer cells is associated with an increase in the expression of PMCA4 (16,17). However, to function as efflux pumps, PMCA4 needs to be located on the plasma membrane, which, we confirmed, is an early event in the differentiation of HT-29 colon

cancer cells and precedes the maximal increase in PMCA4 expression. Despite this being an early event in the differentiation of HT-29 cells, the increased levels of PMCA4 are not sufficient to induce the differentiated phenotype since there was no induction in the expression of the differentiation marker ALPI in PMCA4 overexpressing HT-29 cells.

Consistent with increased expression during the differentiation of colon cancer cells, there was a reduction in the level of PMCA4 calcium pumps in some colon tumors, similar to the downregulation of the endoplasmic reticulum calcium pump SERCA3 in colorectal cancer (8,10,11). PMCA4 downregulation may be an early event in colon cancer, since PMCA4 was downregulated even in benign colon tumors. PMCA4 downregulation may be characteristic of adenoma formation and/or growth and may persist through the stepwise formation to the colon adenocarcinoma (33). SERCA3 downregulation is also suggested to be an early event in colon cancer formation linked with the APC/ β -catenin/T-cell factor 4 -signaling pathway (8). Thus, the remodeling of the calcium signal may be an early event in colon tumorigenesis and provide the cell with a growth advantage. Remodeling via changes in SERCA3 and PMCA4 may augment changes in calcium influx by reducing the ability to sequester calcium into internal stores and by reducing extrusion across the plasma membrane, respectively. Alterations in calcium influx channels in colon cancer include the calcium permeable TRPM8 ion channel (5) and MS4A12, described as a store-operated calcium channel that promotes tumorigenic processes in colon cancer cells (34).

Downregulation of PMCA4 via siRNA changed the response of the cell to ATP and neurotensin by increasing the peak intracellular calcium level, even in the presence of maintained PMCA4 expression. Physiologically, augmentation of calcium may promote the cell's

response to proliferative stimuli. Neurotensin is a promoter of colon cancer cell proliferation (35), and colon tumor cells with downregulated PMCA4 would experience an increase in $[Ca^{2+}]_i$ in response to neurotensin (36). Combined with the ability of the *Wnt/APC*-signaling pathway to activate neurotensin receptor 1 (NT1) (37), PMCA4 downregulation would augment the cellular response to NT1 activation.

Pronounced and prolonged elevation in $[Ca^{2+}]_i$ can be a trigger for cell death (29). However, despite the increased agonist-stimulated peak $[Ca^{2+}]_i$ after PMCA4 knockdown, PMCA4 knockdown was not associated with a loss of cellular viability or increased sensitivity to TRAIL and/or CCCP-mediated cell death. Such a response is consistent with other efflux pumps in the cell acting to prevent a sustained increase in cellular calcium and is probably due to the presence of PMCA1 (16). Hence, the level of PMCA downregulation in colon cancer cells appeared to be more consistent with the promotion of proliferative calcium signals than cell death calcium signals. There may be some apoptotic stimuli that are particularly sensitive to PMCA4 downregulation (38), due to the nature of the calcium signal and/or the pathways involved, and this could be the focus of future studies. However, our work shows that PMCA4 inhibition in HT-29 colon cancer cells is not associated with a generalized increase in cell death.

Reversing the remodeling of calcium efflux in the colon cancer by overexpressing PMCA4 in HT-29 cells and thus increasing calcium efflux, reduced cell proliferation and the expression of the immediate early gene *FOS*. This was not associated with compensatory changes in PMCA1 transcription. Consistent with the opposite role, when CACNA1E, a calcium influx channel subunit, is overexpressed in HEK-293 cells the reverse effect is seen, with increased *FOS* expression, which suggests increased cell proliferation (21). In keeping with PMCA4 being a negative regulator of *FOS* mRNA expression in colon cancer cells, overexpression of human PMCA4b in transgenic rats almost completely abolishes *Fos* induction by endothelin-1 (39).

Because of its ubiquitous expression, PMCA4 was thought to be important in global rather than specific processes, especially compared with pump isoforms with a more restricted tissue distribution such as PMCA2 and PMCA3 (15). Evidence that is more recent suggests that PMCA4 does have specific cellular functions. For example, PMCA4 is important for sperm function with PMCA4-knockout mice having impaired sperm motility and being sterile (40). Breast cancer cell lines have a reduced PMCA4 expression, whereas PMCA2 and PMCA1 expression is increased (23,24). The spatial distribution of PMCA4 may be particularly important since PMCA4-mediated Ca^{2+} efflux in smooth muscle is dependent on PMCA4's localization to caveolae plasma membrane invaginations (41). The importance of PMCA4 localization to caveolae may also be significant in colon cancer, given that caveolin 1 levels are downregulated in human colon tumors (42) and re-expression of caveolin 1 in HT-29 cells reduces their tumorigenicity in nude mice (42). Hence, reduced PMCA-mediated calcium efflux in colon cancer may be a combination of reduced transcription of PMCA, disrupted plasma membrane localization and reduced caveolin 1 levels.

Collectively, these studies show that a remodeling of calcium transporter expression is a key aspect of the early stages of colon cancer development. The remodeling of calcium efflux involves a downregulation of PMCA4, which augments responses to proliferative stimuli without compromising cellular viability. Reduced PMCA expression would be a powerful augmentor of calcium influx pathways that are upregulated in colon cancer. Increasing calcium pump-mediated Ca^{2+} efflux and/or inhibition of calcium influx pathways augmented in colon cancers may offer a potential future therapeutic strategy by reversing the calcium remodeling that occurs in some colon tumors.

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