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Collard, T. J., Fallatah, H. M., Greenhough, A., Paraskeva, C., & Williams, A. C. (2020). BCL–3 promotes cyclooxygenase–2/prostaglandin E_2 signalling in colorectal cancer. *International Journal of Oncology*, *56*(5), 1304-1313. https://doi.org/10.3892/ijo.2020.5013

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BCL-3 promotes Cyclooxygenase-2/Prostaglandin E_2 signalling in colorectal cancer.

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Abbreviations:

BCL-3, B-cell Chronic Lymphocytic 3 Leukaemia (B-CLL)
COX, cyclooxygenase
CRC, colorectal cancer
NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells
PGE_2, prostaglandin E2
siRNA, small interfering RNA
TNF-α, tumour necrosis factor alpha
IL-1β, interleukin one beta

Key words: COX-2, PGE_2, BCL-3, colorectal cancer, Hallmarks of Cancer

Running title: Collard et al: BCL-3 promotes COX-2/PGE_2 signalling

Abstract:

First discovered as an oncogene in leukaemia, recent reports highlight an emerging role for the proto-oncogene BCL-3 in solid tumours. Importantly, BCL-3 is highly expressed in >30% of colorectal cancers and reported to be associated with poor prognosis. However, the mechanism by which BCL-3 regulates tumorigenesis in the large bowel is yet to be fully elucidated. Here we show for the first time that depleting BCL-3 can suppress cyclooxygenase-2 (COX-2)/Prostaglandin E_2 (PGE_2) signalling in colorectal cancer cells, a pathway known to drive many of the hallmarks of cancer.

RNAi-mediated suppression of BCL-3 expression decreased COX-2 expression in colorectal cancer cells both at the mRNA and protein level. This reduction in COX-2 expression led to a significant and functional reduction (30-50%) in the amount of pro-tumorigenic PGE_2 produced by the cancer cells, as shown by enzyme linked immunoassay and medium exchange experiments. In addition, inhibition of BCL-3 expression also significantly suppressed cytokine-induced (TNF-α or IL-1β) COX-2 expression. Taken together, this report identifies a novel role for BCL-3 in colorectal cancer and suggests that expression of BCL-3 may be a key determinant in the COX-2 response to inflammatory cytokines in colorectal tumour cells.

We propose that targeting BCL-3 to suppress PGE_2 synthesis may represent an alternative or complementary approach to using NSAIDs for prevention and/or recurrence in PGE_2-driven tumorigenesis.

Introduction:

Colorectal cancer (CRC) is a major cause of cancer related mortality and is the third most common cause of cancer death in the UK (1, 2). CRC is a multifactorial disease, with both genetic predisposition and environmental factors playing a critical role in its development. Environmental factors that promote colorectal tumorigenesis include chronic inflammation; there are a number of important regulatory pathways linking inflammation and colorectal cancer, such as chemokines, cytokines, NF-κB, cyclooxygenase-2 (COX-2) and its metabolic product prostaglandin E_2 (PGE_2) (3, 4). Deregulation of the COX-2/PGE_2 pathway is considered to be an important, relatively early event in colorectal tumorigenesis, not only because of its role in shaping the tumour microenvironment, but also for enabling many of the hallmarks of cancer (5).

Despite a focus on NF-κB canonical signalling in carcinogenesis (6-9), the NF-κB homodimer subunits NF-κB1 (p50/p50) and NF-κB2 (p52/p52) have become the subject of intense interest as they represent an alternative mode of NF-κB activation (the atypical NF-κB pathway; reviewed in (10)). Although they function as transcriptional repressors due to their lack of transactivation domains (TAD), they can bind transcriptional co-activators via their C-terminal ankyrin repeats (involved in protein-protein interactions) and therefore play a direct role in positively or negatively regulating NF-κB target genes (11). First identified in subgroup of B-cell chronic lymphocytic leukaemias (12, 13), BCL-3 selectively binds to the NF-κB homodimers and depending on the nature of the stimuli can function as either a co-activator or as an inhibitor of NF-κB (14-17).

BCL-3 has been found to be widely expressed in solid tumours (18); in particular, elevated levels of BCL-3 and p52 homodimers have been linked to immortalized human breast epithelial cells (19) and BCL-3 has been proposed as a link between STAT3 signalling and NF-κB in

metastatic breast cancer (20). Furthermore, it has been shown that BCL-3 can promote colorectal tumorigenesis by increasing colorectal cancer cell survival through AKT activation (21), through stabilizing c-MYC protein via ERK activation and by promoting the cancer stem cell phenotype by enhancing β-catenin signalling (22, 23).

Importantly, BCL-3 expression is reported to be de-regulated in colorectal cancer tissue (24); high BCL-3 protein expression associated with poor prognosis in colorectal cancer patients (22). Cytoplasmic localisation of BCL-3 has also been suggested as a potential early diagnostic marker in colorectal cancer (25). Furthermore, BCL-3 expression is up-regulated by key cytokines including tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (18, 26, 27), potentially representing a novel mechanism linking inflammation and cancer.

Given the importance of COX-2/PGE_2 signalling in colorectal tumorigenesis, it is perhaps surprising that the impact of BCL-3 on the expression of COX-2/PGE_2 signalling in colorectal cancer has not been investigated before. In this study we show that depleting BCL-3 expression can inhibit the COX-2/PGE_2 pathway and that BCL-3 could be a key determinant in the COX-2 response to inflammatory cytokines in colorectal tumour. We suggest that targeting BCL-3 to suppress PGE_2 synthesis may be important in preventing early tumour development and tumour recurrence, making BCL-3 an attractive target for cancer prevention either as an alternative or as a complementary approach to using conventional COX inhibitors (non-steroidal anti-inflammatory drugs including aspirin).

Materials and Methods

Cell line and cell culture conditions

The human colorectal adenocarcinoma-derived cell line HCA7 was a kind gift from Dr Susan Kirkland, (Imperial College, London UK); cells were mycoplasma tested on receipt (Mycoalert Plus mycoplasma detection kit, Lonza, Basal, Switzerland). The human colorectal adenocarcinoma-derived cell line HT-29 and human rectal adenocarcinoma-derived cell line SW837 were obtained from the American Type Culture Collection (ATCC, MD, USA, Cat. No. HTB-38 and CCL-235 respectively) and mycoplasma tested on receipt. All experiments were carried out within 6 passages and cells were routinely characterised (see below). The human colorectal adenocarcinoma-derived HCT116, HCT15, SW480, SW620, LOVO, LS174T and specifically rectal SW1463 cell lines were obtained from the American Type Culture Collection (ATCC, MD, USA, Cat. No. CCL-247, CCL-225, CCL-228, CCL-227, CCL-229, CL-118 and CCL-234 respectively), cells were mycoplasma tested on receipt and experiments carried out within 6 passage of receiving. All cell lines were maintained as previously described (28). Cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Thermo Fisher Scientific, UK), supplemented with 10% fetal bovine serum (FBS), 2mM glutamine (Gibco, Thermo Fisher Scientific, UK), 100U/ml penicillin and 100μg/ml streptomycin (Gibco, Thermo Fisher Scientific, UK). The human colorectal adenoma- PC/AA/C1, S/AN/C1, S/RG/C2 and transformed adenoma- PC/AA/C1/SB10 cell lines were derived in this laboratory and grown as described previously (29, 30). Growth medium was DMEM supplemented with 20% FBS, 1μg/ml hydrocortisone sodium succinate, 0.2 units/ml insulin, 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin. 24h conditioned medium (CM) was harvested from BCL-3 siRNA, COX-2 siRNA or negative control transfected HCA7 cells, filtered and used to culture S/RG/C2 adenoma for 72h (CM was refreshed every 24 hours). Add back experiments were carried out using 1μM of dimethyl

PGE2 (Sigma-Aldrich, Gillingham, UK) to the CM prior to culture. All cell lines were routinely checked for microbial contamination (including mycoplasma) and molecularly characterised using an "in house" panel of cellular and molecular markers to check that cell lines have not been cross contaminated (every 3-6 month). Stocks were securely catalogued and stored; passage numbers strictly adhered to preventing phenotypic drift.

Determining percentage of floating cells as a measure of apoptosis.

Growth medium from individual flasks was collected and the floating cells counted. Attached cell yield was determined by trypanising and counting the number of viable cells from the same flask. The floating cells are represented as a proportion of the total cell population (floating and attached), accepted as a measure of cell death (31).

RNA interference

Cells were reverse transfected using Lipofectamine RNAiMAX (Invitrogen, Thermo Fisher, UK), as per manufacturer's instructions, with small interfering RNAs (siRNAs final concentration 50nM) from Dharmacon (Horizon Discovery, Cambridge, UK) targeting BCL-3 (individual sequences) or COX-2 (pooled siRNAs) (21). Cells were incubated overnight at 37°C, before being medium changed onto fresh growth medium.

Reverse Transcription (RT)

Total RNA was extracted from cells using Tri-Reagent (Sigma-Aldrich, Gillingham, UK), a RNeasy mini kit (Qiagen, Manchester, UK) was utilised according to manufacturer's instructions with an additional on-column DNase digestion (RNase-Free DNase Set; Qiagen, Manchester, UK), prior to cDNA synthesis. The RNA concentration of the sample was measured using a NanoDrop (Thermo Scientific, Karlsruhe, Germany).

Complementary DNA (cDNA) was synthesised from 2µg of RNA by reverse transcription (RT), using the RNA-dependent DNA polymerase, moloney murine leukaemia virus (M-MLV)

reverse transcriptase, (Promega, Southampton, UK). A second tube, in the absence of the reverse transcriptase was synthesised as a negative control, no RT. The samples were diluted further giving a final concentration of 10ng/µL.

Real-Time Quantitative-PCR (RT-qPCR)

Having achieved optimisation of primers and ensured the annealing temperature gave a near 100% efficient amplification per cycle, RT-qPCR was performed as previously described (22), using SYBR Green PCR Mix (Qiagen, Manchester, UK) and the following Qiagen QuantiTect primers (Manchester, UK), at a dilution of 1:10: BCL-3 (QT00040040), COX-2 (QT00040586); with gene expression normalised to housekeeping gene TBP (QT00000721) or HPRT (QT00059066). The QuantiTect primer assays sets are designed to have annealing temperatures of 55°C, a 40-cycle program was performed on a MxPro 3005P Real-time Thermal Cycler (Agilent Technologies, West Lothian, UK); samples carried out in triplicate with one no RT well per condition. Amplification data was analysed using MxPro software (Stratagene, Stockport, UK), by means of the ΔΔCq method (32).

Immunoblotting

Whole-cell lysates were prepared *in situ,* on ice, using 100µl 1x lysis buffer (Cell Signaling Technology, Danvers, MA, USA) with the addition of a Protease Inhibitor Cocktail Tablet (Roche, Basal, Switzerland) per 10ml of lysis buffer. The cell debris was removed by centrifugation for 10 minutes at 18,500g. Protein concentration of the cell lysate was determined using the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hemel Hempstead, UK), as per manufacturer's instructions. Absorption was measured in duplicate at 750nm using an iMark Microplate Absorbance Reader (Bio-Rad, Hemel Hempstead, UK). Samples of 100µg total protein concentration were prepared in a volume of 20µl, adjustments made with distilled

water. 5µl of 5x Laemmli buffer was added to each lysate sample and boiled together for 5 minutes.

Mini-Protean 3 Electrophoresis Cells (Bio-Rad, Hemel Hempstead, UK) were used to cast 9% acrylamide resolving gels, using 1.5mm spacers. Gels were run at 100V for approximately 15 minutes permitting the samples to move through the stack, before voltage was increase to 180V for approximately an hour or until the blue dye front had migrated through the gel. Following separation of the protein samples using SDS-PAGE the proteins were transferred onto Immobilon-P, a polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The gel and membrane were then assembled into a Transblot Cell (Bio-Rad, Hemel Hempstead, UK), a voltage of 100V was applied for 1.5 hours. The membrane was blocked in 5% (w/v) milk blocking buffer for a minimum of 1 hour before being subjected to immunoblotting. as previously described (33), using antibodies diluted in 0.5% (w/v) milk dilution buffer: rabbit polyclonal anti-BCL-3 (23959-1-AP, Proteintech, UK), goat polyclonal anti-COX-1 (sc-1752; Santa Cruz Biotechnology, CA, USA), goat polyclonal anti-COX-2 (sc-1745; Santa Cruz Biotechnology, CA, USA) or rabbit polyclonal anti-15-PGDH (ab37148; Abcam, Cambridge, UK). The membranes were incubated overnight at 4°C, after which they were given three 10 minute washes in Tris-Buffered Saline - 0.1% (v/v) Tween 20 (TBS-T); following incubation with an appropriate horseradish peroxidise- (HRP-) conjugated secondary antibody diluted in 0.5% (w/v) milk dilution buffer. After an hour incubation at room temperature with rotation, three further 10-minute washes in TBS-T were completed and the membrane rinsed in distilled water prior to detection by chemiluminescence (LumiGLO Peroxidase Chemiluminescence Substrate, KPL, Gaithersburg, MD, USA). The signal was detected by film developed in a Compact X4 Film Processor (XOgraph Imaging Systems Ltd., Stonehouse, UK); the length of time films were exposed depended on the strength of the signal

(between 1-20 minutes). Equal loading was confirmed with monoclonal mouse anti-α-tubulin (T9026; Sigma, Gillingham, UK).

Blots were quantified using Image J software (NIH, Maryland, USA), this densitometry tool was used to measure the change in intensity of individual western blot bands.

PGE_2 Assay

PGE_2 released by cells into culture media was quantified, using a PGE_2 enzyme immunoassay solid well kit (Cayman Chemical Europe, Estonia), that uses a high-affinity PGE_2 monoclonal antibody for quantification of PGE_2. Medium collected from 70% confluent HCA7 cells was snap frozen in liquid nitrogen (−196°C) and stored at −70°C. PGE_2 levels were determined using the immunoassay kit, as described previously (34). Cells were pre-treated with TNF-α (100ng/ml, Insight Biotechnology Ltd., UK) for 16h or NS-398 (20μM/ml, Sigma-Aldrich, Gillingham, UK) for 24 hours as required. Samples were performed in triplicate.

Statistical analysis

Statistical analysis, ANOVA, student and one sample t-tests, were performed using GraphPad Prism (GraphPad Software Inc, California, USA), and represented as *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; NS=non-significant. Results are expressed as mean values ± standard error of the mean (SEM) where a minimum of three independent experiments were performed. Where a single experiment was performed the mean of triplicate readings ± standard deviation (SD).

Results

Suppression of BCL-3 expression decreases COX-2 expression in colorectal cancer cells.

This study was based on the observation that suppression of BCL-3 consistently repressed *PTGS2* expression in a number of human colorectal cancer cell lines (data from focused mini arrays, not shown). The purpose of this study was to determine whether BCL-3 regulates COX-

2/PGE_2 signalling in colorectal cancer. For these studies HCA7 human colorectal cancer cells (high endogenous COX-2 expression, high BCL-3 expression (Figure 1A) were transfected with BCL-3 siRNA and the expression of COX-2 was determined (Figure 1B). Cells were transfected with 50nM BCL-3 siRNA or non-targeting control. To confirm the specificity of the siRNA and to control for off-target effects, BCL-3 expression was silenced using four separate siRNA sequences (A, B, C and D). Importantly, transfection with the all four separate siRNA sequences led to a reduction in the level of COX-2 protein (Figure 1B). The expression of both BCL-3 and COX-2 proteins in HCA7 cells was assessed at 24, 48 and 72h post-transfection (Figure 1C). Western blot analysis showed that the level of COX-2 protein was reduced following suppression of BCL-3 at all-time points (Figure 1C(i)). BCL-3 and COX-2 mRNA was evaluated by RT-qPCR. Results are presented as fold change of the negative siRNA transfected levels (Figure 1C(ii)). Suppression of BCL-3 expression resulted in a greater than 60% reduction in COX-2 mRNA, confirming that down-regulation of BCL-3 in colorectal cancer cells decreases both the COX-2 mRNA and protein expression.

To determine whether BCL-3 could regulate COX-2 expression in other colorectal cancer cell lines, HT-29 (which has an intermediate level of endogenous COX-2 protein expression, Figure 1D) and the SW837 rectal cancer cell line (low endogenous COX-2 protein expression, Figure 1E) were investigated, Transfection with BCL-3 siRNA also led to suppression of COX-2 mRNA and protein in both cell lines.

Suppression of BCL-3 expression decreases COX-2/PGE_2 signalling.

To confirm that the decrease in COX-2 expression on transfection with BCL-3 siRNA resulted in reduced COX-2 activity, PGE_2 levels were measured in the culture medium. (PGE_2 is a cyclooxygenase derived metabolite of arachidonic acid (5).) HCA7 cells were selected for this experiment as the high endogenous COX-2 expression allows PGE_2 to be measured directly by

enzyme linked immunoassay (EIA) (Figure 2A). COX-1 and 15-PGDH protein expression were also assessed by western blotting 72h after BCL-3 siRNA transfection (Figure 2B); to ensure that other enzymes which control PGE_2 levels were not also regulated. Furthermore, cells were treated with NS-398 (20μM; a dose selective for COX-2 inhibition (35)) to further validate that changes in PGE_2 detected in the cell culture medium were due to COX-2 and not COX-1 activity (Figure 2A). The results show that there was significant inhibition (p-value=0.048) of PGE_2 production when BCL-3 expression is suppressed; there was an approximate 30% decrease in basal PGE_2 production on BCL-3 siRNA transfection when compared to the negative siRNA control. Importantly, the production of more than 90% of the PGE_2 was blocked by the COX-2 selective inhibitor NS-398 (Figure 2A). Finally, COX-1 and 15-PGDH levels were not regulated on transfection with the BCL-3 siRNA (Figure 2B). Taken together, these findings show that the observed decrease in PGE_2 production in the BCL-3 siRNA transfected cells is due to a loss of COX-2 activity.

We next wanted to investigate whether the regulation of PGE_2 observed by suppression of BCL-3 could affect the survival of other tumour cells. For this we carried out conditioned medium exchange experiments, using a PGE_2 sensitive adenoma derived cell line (S/RG/C2). Previous studies have shown that PGE_2 promotes the growth of S/RG/C2 (36). Conditioned medium (CM) from the BCL-3 siRNA, COX-2 siRNA or negative control transfected HCA7 cells was harvested and used to culture the S/RG/C2 adenoma derived cell line (refer to methods). It is important to note the number of HCA7 cells is not affected by BCL-3 or COX-2 suppression during the preparation of conditioned medium (over the 72h period, Supplementary Figure 1A); results are summarised in Figure 2C. Treating S/RG/C2 cells with CM from the BCL-3 siRNA transfected HCA7 cells resulted in a significant increase (p=0.015) floating cells which is a measure of cell death (31). Importantly, this cell death could be rescued by addition of 1μM of dimethyl PGE_2 (Sigma-Aldrich, Gillingham, UK) to the CM prior to

culture. In addition, the cell death was similar to that seen in cells grown in CM from HCA7 transfected with the COX-2 siRNA (Figure 2C). These findings suggest that the changes in PGE_2 secretion caused by silencing BCL-3 can affect the survival of PGE_2 sensitive tumour cells.

In summary, experiments show that inhibition of BCL-3 expression suppresses COX-2 and significantly reduces the amount of potentially pro-tumorigenic PGE_2 produced by the cancer cells.

Induction of COX-2 expression by TNF-α or IL-1β can be partially blocked by suppressing BCL-3 expression in colorectal cancer cells.

COX-2 protein has been identified as an immediate-early response gene that although normally absent from most cells, may be induced at sites of inflammation in response to cytokines such as TNF-α and IL-1β (37). These cytokines have also been shown to increase COX-2 expression in colorectal cancer cells (3, 38, 39). To address whether targeting BCL-3 could reduce the induction of COX-2 in response to inflammatory cytokines, HCA7 and HT-29 cells were treated with 100ng/ml of TNF-α for up to 72h (Figure 3) whilst SW837 were treated with 10ng/ml IL-1β (Figure 4; SW837 are less responsive to TNF-α). BCL-3 and COX-2 protein expression were measured over 72h, the concentration of TNF-α and IL-1β used had been previously optimized (40). The first thing to note is that BCL-3 protein levels increase after 2h with exposure to TNF-α (Figure 3A) reaching a peak at 4h-8h treatment and returning to basal levels by 72h. COX-2 expression followed BCL-3 induction; COX-2 is induced at 4h-8h, and remained high for 24h-48h treatment with TNF-α, consistent with it being regulated by BCL-3 (Figure 3A). Notably, the increased expression of COX-2 in response to TNF-α treatment (shown in Figure 3B at 4h) was found to be partially blocked following the suppression of BCL-3 in both HCA7 and HT-29 cell lines. Importantly, suppression of BCL-3 also inhibited the PGE_2 produced by the HCA7 cells in response to TNF-α treatment (Figure 3C). Similar

findings were observed in the SW837 cells treated with 10ng/ml IL-1β (Figure 4A-D). Due to the low protein expression in the SW837 cells, expression of both BCL-3 and COX-2 were also measured using RT-qPCR (Figure 4B and D). Again, there is rapid induction of BCL-3 (within 2h) after IL-1β treatment, followed by a more prolonged induction of COX-2 (Figures 4A[protein], 4B[mRNA]). In addition, the increase in COX-2 expression 2h-4h after IL-1β treatment was partially blocked following suppression of BCL-3 by siRNA (Figure 4C[protein], 4D[mRNA]). This data shows that inhibition of BCL-3 expression not only reduced basal COX-2 but also significantly supresses cytokine induced COX-2 expression. These results suggest that expression of BCL-3 may be an important determinant in the COX-2 response to inflammatory cytokines.

Discussion:

Discovered as an oncogene in leukaemia, BCL-3 has been found to be overexpressed in a range of solid tumours including breast (19), prostate (41), endometrial (42) and nasopharyngeal (43). The importance of BCL-3 in colorectal carcinogenesis is supported by both clinical and mechanistic studies. Puvvada and colleagues were the first to show that high nuclear BCL-3 correlated with poor prognosis in colorectal cancer patients (24), and Saamarthy *et al*. propose BCL-3 cellular localisation as a marker for early diagnosis in colorectal cancer (25). Both studies report that at least 30% of colorectal tumours had increased nuclear expression of BCL-3 (24, 25). More recently high BCL-3 expression has been associated with worse survival in CRC (22)

Importantly, there are a number of studies that begin to shed light on the mechanism(s) by which BCL-3 can promote colorectal carcinogenesis; we have shown that BCL-3 expression promotes AKT mediated cell survival and drives colorectal tumour growth in vivo (21), BCL-3 has also been reported to increase the stability of c-MYC via ERK activation (23). In addition,

BCL-3 has been implicated in promoting tumorigenesis through inhibition of DNA damage induced p53 activation by up-regulating MDM2 expression (44), and to induce cyclin D and cell cycle progression (45). Most recently, we have shown that BCL-3 promotes the cancer stem cell phenotype by enhancing β-catenin signalling in colorectal tumour cells (reviewed in (46)).

Given the importance of increased COX-2 in colorectal tumorigenesis, our novel finding that BCL-3 regulates *PTGS2* gene expression and COX-2/PGE_2 signalling identifies a new mechanism to support the oncogenic actions of BCL-3 in colorectal cancer. Although not required for COX-2 expression, we propose that nuclear BCL-3 acts as a transcriptional co-factor (as recently proposed for β-catenin-dependent gene targets for example, (22)). It remains to be determined whether BCL-3 enhances NF-κB-dependent COX-2 regulation or acts via recruiting other co-regulators, such as Pirin, Tip60, Jab1 and Bard1 (47). The ability of BCL-3 to regulate PGE_2 production is of particular importance in light of recent studies; not only does PGE_2 promote expression of the intestinal cancer stem cell marker LGR5 (36), but PGE_2 production by colorectal cancer cells (as well as melanoma and breast cancer cells) suppresses tumour immunity and fuels tumour promoting inflammation (48). COX-dependent immune evasion has been shown to be critical for tumour growth in colorectal cancer mouse models, COX-2 being reported to be responsible for the majority of circulating PGE_2 (37, 48). A limitation of the study is that TNF-α and IL-1β are only two of the upstream controllers of COX-2 activity, whether BCL-3 expression regulates others remains to be determined. Furthermore, the potential to affect many of the downstream targets of COX2/PGE2 signalling remains to be discovered (5).

Here we present evidence that targeting BCL-3 expression could suppress COX-2/PGE_2 signalling, which not only impacts the growth of other tumour cells (as shown here as PGE_2

drives the hallmarks of cancer (5)), but could potentially enhance an immune response to the cancer.

Taken together and given the importance of these pathways in tumour progression, targeting the BCL-3 protein-protein interactions (via disruption of the ankyrin repeat domain for example) remains a priority. It is hoped that the inhibitors of the BCL-3 pathway currently under development (Clarkson RWE, Cardiff University, UK) will not only suppress tumour cell survival and block tumour growth, but also increase anti-cancer immunity through suppressing COX-2/PGE_2 signalling. Hence, these findings suggest BCL-3 inhibitors could be used as an alternative or complementary approach to using NSAIDs for prevention and/or recurrence in PGE_2-driven tumorigenesis (49), improving the long-term prognosis for colorectal cancer patients.

Acknowledgements

We thank the Medical Research Council for providing an Infrastructure Award to establish the School of Medical Sciences Wolfson Bioimaging Facility and the staff for support and guidance. We also thank members of the Cancer Research UK Colorectal Tumour Biology Group for useful discussion.

Funding: This work was funded by a Cancer Research UK programme grant (C19/A11975) (TJC, AG, CP, ACW), a Saudi Government studentship (S6692) (HF) and support from the John James Bristol Foundation (all).

Data and Material availability

All data has been generated and curated according to the University of Bristol's 'Guidelines on Good Research Practice'; we fully support a commitment to data sharing to maximise the

impact of our work. Reagents will be made freely available to the research community upon request.

A**uthors' contributions**

Conception and design [ACW, HF, TJC, CP, AG], Development of methodology [ACW, HF, TJC, AG], Acquisition of data [ACW, HF, TJC, CP, AG], Analysis and interpretation of data [ACW, HF, TJC, CP, AG] Writing [TJC, ACW] review and/or revision of the manuscript [All] Administrative, technical, or material support [TJC] Study supervision [ACW, CP].

Competing interests: “The authors declare that they have no competing interests”

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Figure Legends

Figure 1. BCL-3 knockdown reduces COX-2 expression. (A) The endogenous levels of BCL-3 and COX-2 in a panel of colorectal adenoma and carcinoma derived cell lines. Colorectal adenoma- (PC/AA/C1, S/AN/C1, S/RG/C2), transformed adenoma- (PC/AA/C1/SB10) and colorectal adenocarcinoma- (HT-29, HCA7, HCT116, HCT15, SW480, SW620, LOVO, LS174T, and specifically rectal SW837 and SW1463) derived cell lines were grown to approximately 70% confluence before collection of total protein for western blot analysis. Equal loading was confirmed by α-tubulin. **(B)** Western blot for BCL-3 in HCA7 cells to validate BCL-3 siRNA sequences as well as the BCL-3 antibody. The expression level of BCL-3 was measured in HCA7 cells 72h after knocking down BCL-3 using four separate BCL-3 siRNAs sequences (A, B, C and D). The western blot is representative of two independent experiments. Probing for α-tubulin confirmed equal loading. Sequence A was used in all subsequent experiments **(C-E) (i)** Western blot analysis and **(ii)** real-time quantitative-PCR (RT-qPCR) mRNA analysis in **(C)** HCA7 **(D)** HT-29 and **(E)** SW837 cells transfected with 50nM of control siRNA or BCL-3 siRNA. Of note, the visible upper non-specific band is the result of a long exposure time in order to detect low endogenous COX-2 levels in the SW837 cells. **(i)** BCL-3 siRNA transfected cells were harvested for total protein at 24h, 48h, and 72h post-transfection and BCL-3 and COX-2 levels were assessed by western blot. α-tubulin was used to assess loading. The results represent three independent experiments. **(ii)** From parallel flasks, real-time quantitative PCR was carried out at 24h and 48h post-transfection of BCL-3 siRNA, relative mRNA quantity of BCL-3 and COX-2 are presented as a fold change of the control negative siRNA, normalised to one. All mRNA values were normalised to the housekeeping gene *TBP* or *HPRT* (data represent four independent experiments, n=4), one sample t-test ± SEM *P≤0.05, **P≤0.01 ***P≤0.001.

Figure 2. BCL-3 knockdown suppresses PGE_2 production in the HCA7 cell line. (A) Data represents the amount of PGE_2 produced (as measured by enzyme linked immunoassay, pg/ml per 10^6 cells) by HCA7 cell line and is compared to NS-398 (20μM) treated cells. Cells were transfected with 50nM of control siRNA or BCL-3 siRNA. PGE_2 production was determined 72h after siRNA transfection. Values are the mean of three independent experiments ±SEM, *P≤0.05, NS= Not Significant. **(B)** Western blot shows that the down-regulation of BCL-3 has no effect on COX-1 or 15-PGDH protein levels up to 72h post BCL-3 siRNA transfection. Cells were transfected with 50nM of control siRNA or BCL-3 siRNA, and lysed on ice at 48h and 72h post-transfection. Results are representative of three independent experiments. **(C)** Medium exchange experiments: 24h conditioned medium (CM) was harvested from BCL-3 siRNA, COX-2 siRNA or negative control transfected HCA7 cells and used to culture S/RG/C2 adenoma for 72h (CM was refreshed every 24 hours). Induction of cell death is represented by the number of floating cells as proportion of total cell yield (31). Results are presented as a fold change of the control negative siRNA. 1μM PGE_2 was added to CM in the rescue experiment. Values are the mean of three independent experiments ±SEM. ANOVA was used to determine the statistical significance, n=3, *P≤0.05, **P≤0.01.

Figure 3. TNF-α induced COX-2 protein is partially blocked following the suppression of BCL-3 in siRNA treated cells. (A) Western blotting shows the expression of BCL-3 and COX-2 proteins following TNF-α treatment (100ng/ml) in **(i)** HCA7 and **(ii)** HT-29 cells. The results are representative of three independent experiments. **(B)** Representative western blot shows the expression of COX-2 protein following the suppression of BCL-3 in TNF-α treated cells. **(i)** HCA7 and **(ii)** HT-29 cells were transfected with 50nM of control siRNA or BCL-3 siRNA and treated with TNF-α (100ng/ml) for 4h. BCL-3 and COX-2 protein levels were determined at 72h post-transfection. Equal loading was confirmed by α-tubulin. Graph shows the fold of change of COX-2 protein expression following TNF-α treatment (100ng/ml) for 4h.

The quantification of COX-2 was calculated using Image J software to measure the change in intensity of individual western blot bands, results are presented normalized to the control. Results are mean values from three independent measurements ± SD. **(C)** Data represents the amount of PGE_2 produced pg/ml per 10^6 cells in TNF-α (100ng/ml) treated HCA7 cells. HCA7 cells were transfected with 50nM of control siRNA or BCL-3 siRNA. PGE_2 production was determined 72h after siRNA transfection. Values are the means of three independent experiements ±SEM, *P≤0.05.

Figure 4. IL-1β induced COX-2 protein is partially blocked following the suppression of BCL-3 in siRNA treated SW837 cells. (A) Western blotting shows the expression of BCL-3 and COX-2 proteins following IL-1β treatment (10ng/ml) in SW837 cells. The results are representative of three independent experiments. **(B)** Quantitative real-time PCR was carried out up to 48h post-treatment with IL-1β (10ng/ml). Data presented as a fold change of the untreated control. All mRNA values were normalised to the housekeeping gene *TBP* or *HPRT* Data represent four independent experiments, n=4. **(C)** Representative western blot shows the expression of COX-2 protein following the suppression of BCL-3 in IL-1β treated cells. SW837 cells were transfected with 50nM of control siRNA or BCL-3 siRNA and treated with IL-1β (10ng/ml) for 4h. BCL-3 and COX-2 protein levels were determined at 72h post-transfection. Equal loading was confirmed by α-tubulin. Graph shows the fold of change of COX-2 protein expression following IL-1β treatment (10ng/ml) for 4h. The quantification of COX-2 was calculated using Image J software, to measure the change in intensity of individual western blot bands, and results presented normalized to the control. Results are mean values from three independent measurements ±SD, ***P≤0.001. **(D)** Data represents BCL-3 and COX-2 mRNA expression following transfection with BCL-3 or COX-2 siRNA (50nM). Quantitative real-time PCR was carried out following 2h IL-1β treatment (10ng/ml), 72h post-transfection, results presented as a fold change of the control negative siRNA. All mRNA

values were normalised to the housekeeping gene *TBP* or *HPRT*. Data show a significant reduction in COX-2 mRNA on transfection with BCL-3 siRNA, even after 2h IL-1β treatment (10ng/ml). Transfection with COX-2 siRNA has no effect on levels of BCL-3 expression. Data represents four independent experiments, n=4, ANOVA was used to determine the statistical significance ± SEM, **P≤0.01, ***P≤0.001.

Supplementary Figure 1. Average cell yield of HCA7 cells transfected with BCL-3 or COX-2 siRNA. A(i) HCA7 cells were selected for preparation of conditioned medium (CM) due to high endogenous COX-2/PGE_2 levels. In additon, unlike other colorectal cancer cells tested (21) the cell yield (the number of viable cells) is not significantly changed by suppression of BCL-3 or COX-2 over the 72h period of the experiment**.** Cell yield is expressed as a percentage of the negative siRNA control cells. Values are the mean of three independent experiments ±SEM, ANOVA was used to determine the statistical significance. n=3, NS=non-significant. (ii) Western blot confirming the knock-down of BCL-3 or COX-2 in HCA7 cells transfected with BCL-3 siRNA or COX-2 siRNA respectively, before harvesting the CM. Equal loading was confirmed by α-tubulin. The results represent three independent experiments.

Figure 1

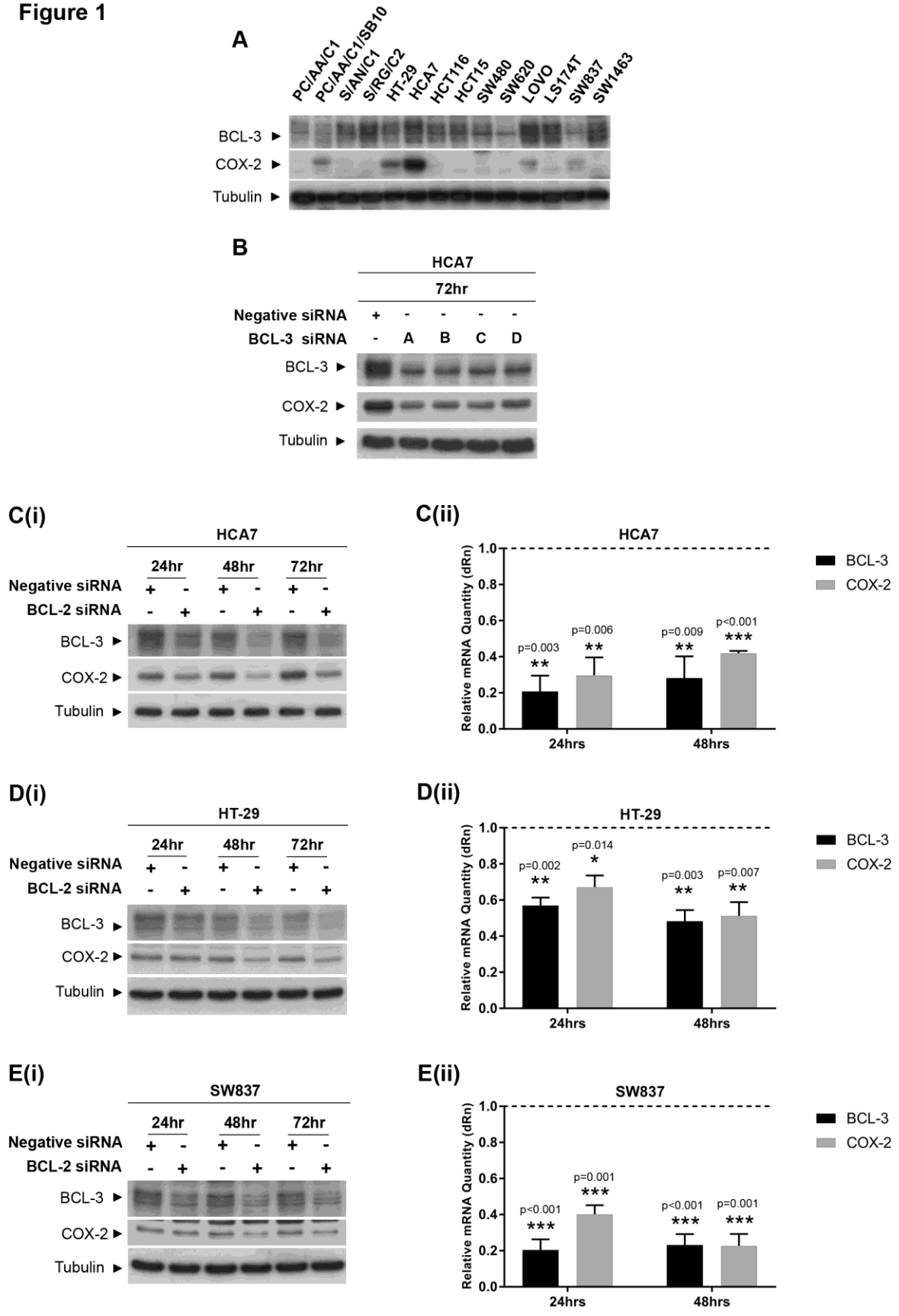

A
PC/AA/C1
PC/AA/C1/SB10
S/AN/C1
S/RG/C2
HT-29
HCA7
HCT116
HCT15
SW480
SW620
LOVO
LS174T
SW837
SW1463
BCL-3 ►
COX-2 ►
Tubulin ►
B
HCA7
72hr
Negative siRNA + - - - -
BCL-3 siRNA - A B C D
BCL-3 ►
COX-2 ►
Tubulin ►
C(i)
HCA7
24hr 48hr 72hr
Negative siRNA + - + - + -
BCL-2 siRNA - + - + - +
BCL-3 ►
COX-2 ►
Tubulin ►
C(ii)
HCA7
Relative mRNA Quantity (dRn)
1.0
0.8
0.6
0.4
0.2
0.0
p=0.003 **
p=0.006 **
p=0.009 **
p<0.001 ***
24hrs
48hrs
BCL-3
COX-2
D(i)
HT-29
24hr 48hr 72hr
Negative siRNA + - + - + -
BCL-2 siRNA - + - + - +
BCL-3 ►
COX-2 ►
Tubulin ►
D(ii)
HT-29
Relative mRNA Quantity (dRn)
1.0
0.8
0.6
0.4
0.2
0.0
p=0.002 **
p=0.014 *
p=0.003 **
p=0.007 **
24hrs
48hrs
BCL-3
COX-2
E(i)
SW837
24hr 48hr 72hr
Negative siRNA + - + - + -
BCL-2 siRNA - + - + - +
BCL-3 ►
COX-2 ►
Tubulin ►
E(ii)
SW837
Relative mRNA Quantity (dRn)
1.0
0.8
0.6
0.4
0.2
0.0
p<0.001 ***
p=0.001 ***
p<0.001 ***
p=0.001 ***
24hrs
48hrs
BCL-3
COX-2

Figure 2

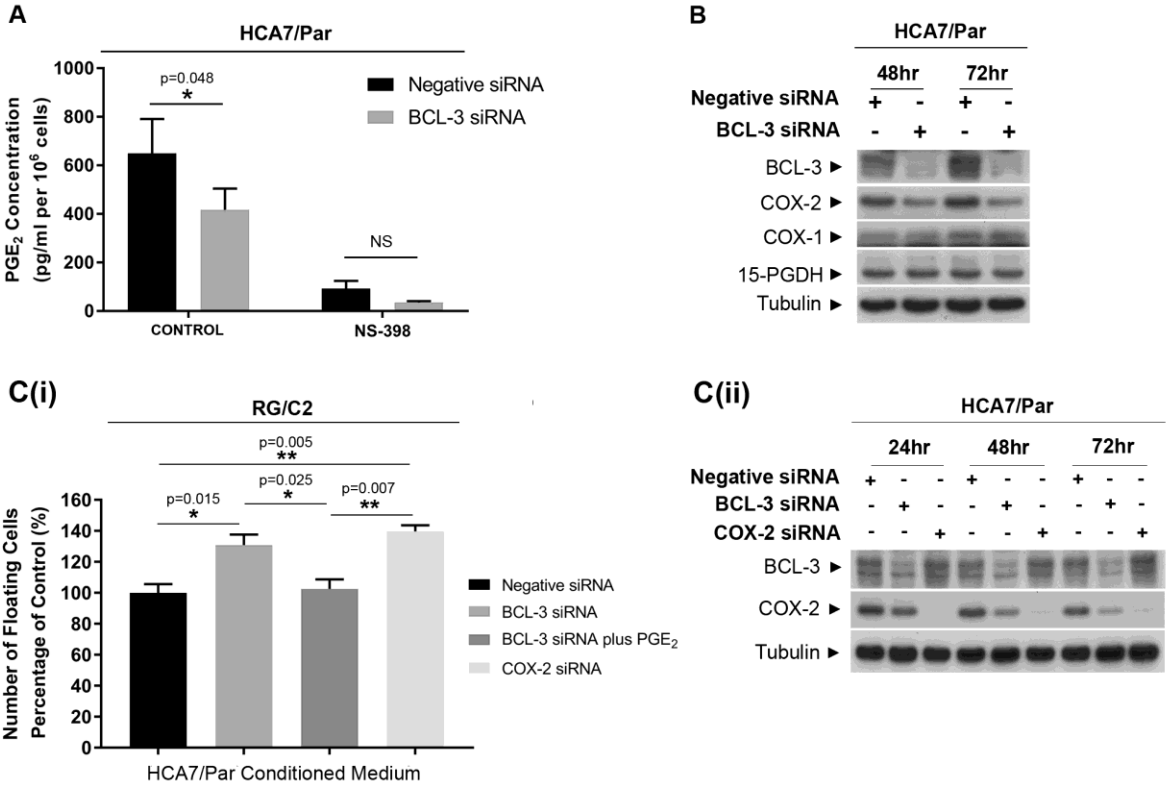

A
HCA7/Par
PGE2 Concentration (pg/ml per 10^6 cells)
1000
800
600
400
200
0
p=0.048
*
NS
Negative siRNA
BCL-3 siRNA
CONTROL
NS-398
B
HCA7/Par
48hr
72hr
Negative siRNA + - + -
BCL-3 siRNA - + - +
BCL-3 ►
COX-2 ►
COX-1 ►
15-PGDH ►
Tubulin ►
C(i)
RG/C2
p=0.005
**
p=0.015
*
p=0.025
*
p=0.007
**
Number of Floating Cells Percentage of Control (%)
160
140
120
100
80
60
40
20
0
Negative siRNA
BCL-3 siRNA
BCL-3 siRNA plus PGE2
COX-2 siRNA
HCA7/Par Conditioned Medium
C(ii)
HCA7/Par
24hr
48hr
72hr
Negative siRNA + - - + - - + - -
BCL-3 siRNA - + - - + - - + -
COX-2 siRNA - - + - - + - - +
BCL-3 ►
COX-2 ►
Tubulin ►

Figure 3

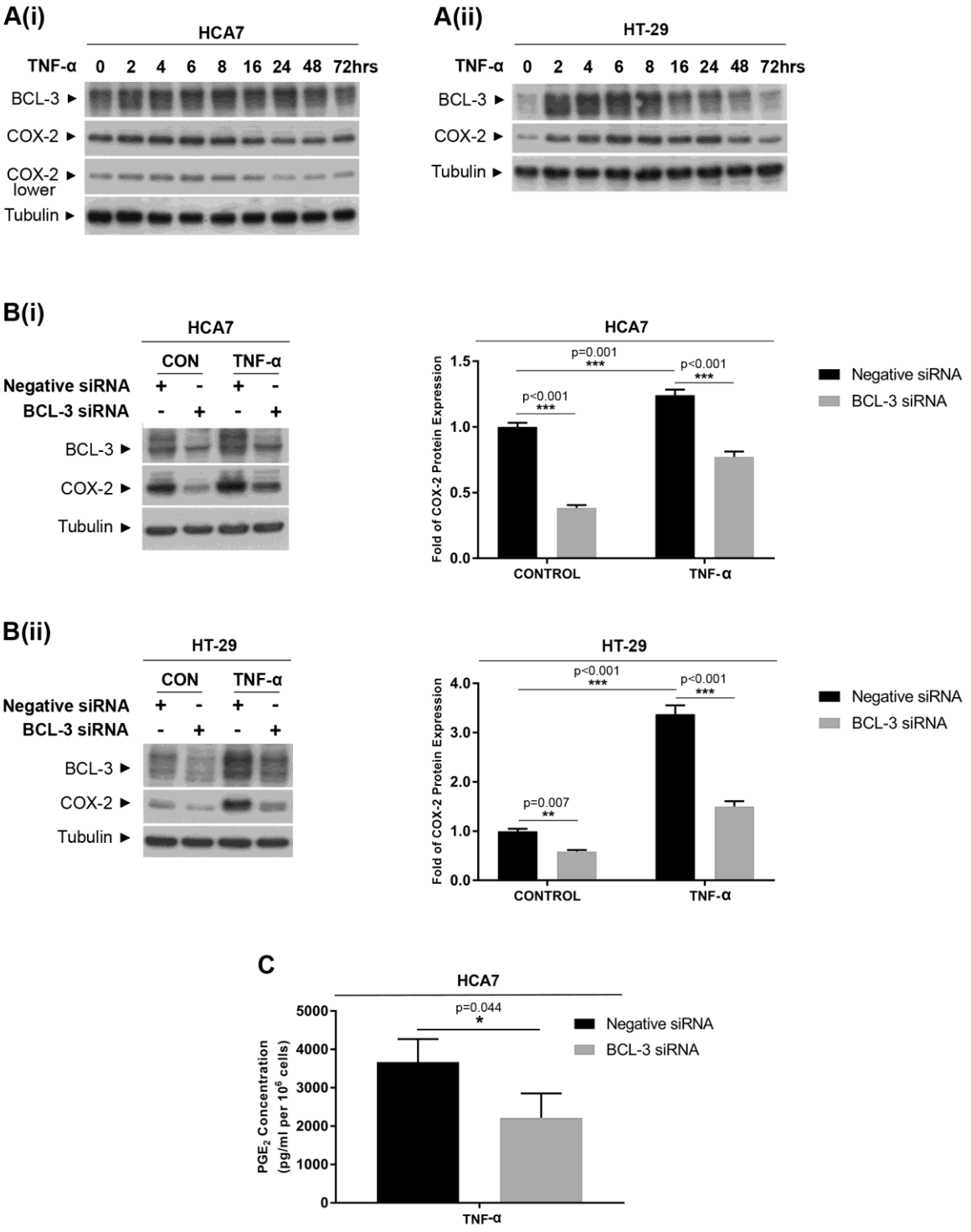
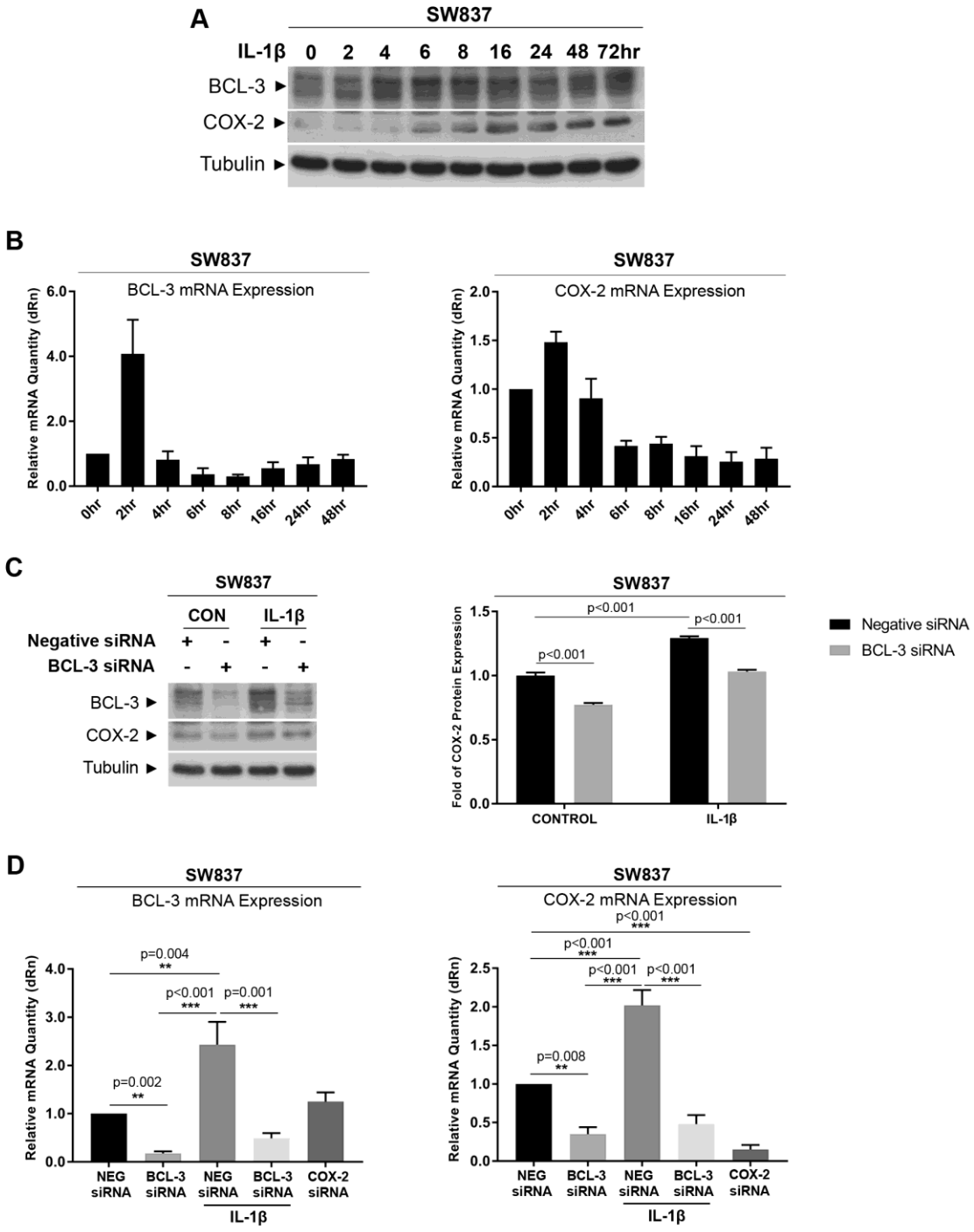
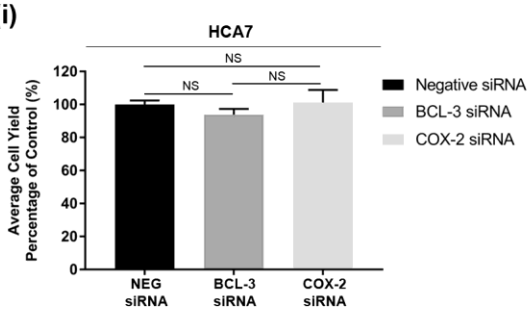
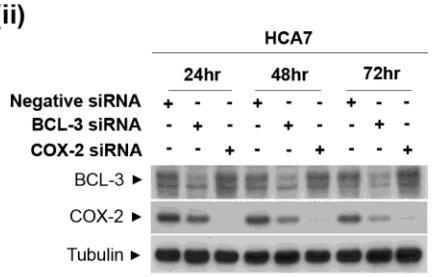

A(i)
HCA7
TNF-α 0 2 4 6 8 16 24 48 72hrs
BCL-3 ►
COX-2 ►
COX-2 lower ►
Tubulin ►
A(ii)
HT-29
TNF-α 0 2 4 6 8 16 24 48 72hrs
BCL-3 ►
COX-2 ►
Tubulin ►
B(i)
HCA7
CON TNF-α
Negative siRNA + - + -
BCL-3 siRNA - + - +
BCL-3 ►
COX-2 ►
Tubulin ►
HCA7
p=0.001 ***
p<0.001 ***
p<0.001 ***
Fold of COX-2 Protein Expression
1.5
1.0
0.5
0.0
CONTROL
TNF-α
Negative siRNA
BCL-3 siRNA
B(ii)
HT-29
CON TNF-α
Negative siRNA + - + -
BCL-3 siRNA - + - +
BCL-3 ►
COX-2 ►
Tubulin ►
HT-29
p<0.001 ***
p<0.001 ***
p=0.007 **
Fold of COX-2 Protein Expression
4.0
3.0
2.0
1.0
0.0
CONTROL
TNF-α
Negative siRNA
BCL-3 siRNA
C
HCA7
p=0.044 *
PGE2 Concentration (pg/ml per 10^6 cells)
5000
4000
3000
2000
1000
0
TNF-α
Negative siRNA
BCL-3 siRNA

Figure 4


A
SW837
IL-1β 0 2 4 6 8 16 24 48 72hr
BCL-3 ►
COX-2 ►
Tubulin ►
B
SW837
BCL-3 mRNA Expression
Relative mRNA Quantity (dRn)
0hr 2hr 4hr 6hr 8hr 16hr 24hr 48hr
SW837
COX-2 mRNA Expression
Relative mRNA Quantity (dRn)
0hr 2hr 4hr 6hr 8hr 16hr 24hr 48hr
C
SW837
CON IL-1β
Negative siRNA + - + -
BCL-3 siRNA - + - +
BCL-3 ►
COX-2 ►
Tubulin ►
SW837
Fold of COX-2 Protein Expression
p<0.001
p<0.001
p<0.001
CONTROL
IL-1β
Negative siRNA
BCL-3 siRNA
D
SW837
BCL-3 mRNA Expression
Relative mRNA Quantity (dRn)
p=0.004
**
p<0.001
***
p=0.001
***
p=0.002
**
NEG siRNA
BCL-3 siRNA
NEG siRNA
BCL-3 siRNA
COX-2 siRNA
IL-1β
SW837
COX-2 mRNA Expression
Relative mRNA Quantity (dRn)
p<0.001
***
p<0.001
***
p<0.001
***
p<0.001
***
p=0.008
**
NEG siRNA
BCL-3 siRNA
NEG siRNA
BCL-3 siRNA
COX-2 siRNA
IL-1β

Supplementary

A(i)



A(ii)