BCL-3 promotes cyclooxygenase-2/prostaglandin E_2 signalling in colorectal cancer

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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 expression is upregulated in >30% of colorectal cancer cases and is reported to be associated with a poor prognosis. However, the mechanism by which BCL-3 regulates tumorigenesis in the large intestine is yet to be fully elucidated. In the present study, it was shown for the first time that knocking down BCL-3 expression suppressed cyclooxygenase-2 (COX-2)/prostaglandin E_2 (PGE_2) signalling in colorectal cancer cells, a pathway known to drive several 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 resulted in a significant and functional reduction (30-50%) in the quantity of pro-tumorigenic PGE_2 produced by the cancer cells, as shown by enzyme linked immunoassays 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, the results of the present study identified a novel role for BCL-3 in colorectal cancer and suggested that expression of BCL-3 may be a key determinant in the COX-2-meditated response to inflammatory cytokines in colorectal tumour cells. These results suggest that targeting BCL-3 to suppress PGE_2 synthesis may represent an alternative or complementary approach to using non-steroidal anti-inflammatory drugs [(NSAIDs), which inhibit cyclooxygenase activity and suppress the conversion of arachidonic acid to prostaglandin], for prevention and/or recurrence in PGE_2-driven tumorigenesis.

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Abbreviations: BCL-3, B-cell chronic lymphocytic leukaemia 3; COX, cyclooxygenase; CRC, colorectal cancer; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; PGE_2, prostaglandin E_2; siRNA, small interfering RNA; TNF-α, tumour necrosis factor α; IL-1β, interleukin-1β

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

Introduction

Colorectal cancer is a major cause of cancer-associated mortality and is the third most common cause of cancer-associated death in the UK (1,2). Colorectal cancer is a multifactorial disease, with both genetic predisposition and environmental factors (such as the microbiota and chronic inflammation) playing important roles in its development (3). There are a number of 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) (4,5). 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 several of the hallmarks of cancer (6).

Despite a focus on NF-κB canonical signalling in carcinogenesis (7-10), 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 (an atypical NF-κB pathway); reviewed in (11). Although the NF-κB homodimer subunits (p50/p50 or p52/p52) function as transcriptional repressors due to their lack of transactivation domains, they can bind transcriptional co-activators via their C-terminal ankyrin repeats (involved in protein-protein interactions) and therefore serve a direct role in positively or negatively regulating NF-κB target genes (12). First identified in a subgroup of B-cell chronic lymphocytic leukaemia (13,14), 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 by either increasing transcriptional activation or through stabilisation of repressive homodimeric complexes, respectively (15-18), reviewed in (19).

BCL-3 has been shown to be widely expressed in solid tumours (20); in particular, elevated levels of BCL-3 and p52 homodimers have been linked to immortalized human breast epithelial cells (21), and BCL-3 has been proposed as a link between STAT3 signalling and NF-κB in metastatic breast cancer (22). Furthermore, it has been shown that BCL-3 can promote colorectal tumorigenesis by increasing survival of colorectal cancer cells through activation of AKT (23), through stabilizing c-MYC protein via ERK activation, and by promoting a cancer stem cell phenotype by enhancing β-catenin signalling (24,25).

BCL-3 expression has been reported to be dysregulated in colorectal cancer tissues (26); high BCL-3 protein expression is associated with a poor prognosis in patients with colorectal cancer (24). Cytoplasmic localisation of BCL-3 has also been suggested as a potential early diagnostic marker in colorectal cancer (27). Furthermore, BCL-3 expression is upregulated by key cytokines including tumour necrosis factor-α (TNF-α) and interleukin-(IL)-1β (20,28,29), potentially representing a novel mechanism linking inflammation and cancer.

Although the importance of COX-2/PGE_2 signalling in colorectal tumorigenesis is well established (6,30,31), the effect of BCL-3 on the expression of COX-2/PGE_2 signalling in colorectal cancer has not been investigated before, to the best of our knowledge. Based on results from focused arrays, it was hypothesized that BCL-3 may be a key determinant of the COX-2 response to inflammatory cytokines in colorectal tumours. Therefore, the aim of the present study was to determine whether suppressing BCL-3 expression inhibited the activity of the COX-2/PGE_2 pathway.

Materials and methods

Cell lines and cell culture. The human colorectal adenocarcinoma-derived cell line HCA7 was a kind gift from Dr Susan Kirkland, (Imperial College, London); cells were mycoplasma tested on receipt (Mycoalert Plus mycoplasma detection kit; Lonza Group Ltd.). 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; cat. nos. HTB-38 and CCL-235, respectively) and mycoplasma tested on receipt. All experiments were performed within 6 passages and cells were routinely characterised (as described below). The human colorectal adenocarcinoma-derived HCT116, HCT15, SW480, SW620, LOVO and LS174T cells, and the specifically rectal cell line SW1463, were all obtained from ATCC (cat. nos. CCL-247, CCL-225, CCL-228, CCL-227, CCL-229, CL-118 and CCL-234, respectively), cells were mycoplasma tested on receipt and experiments were performed within 6 passages of receipt. All cell lines were maintained as previously described (32). Cell lines were grown in DMEM (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS, 2 mM glutamine (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). 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 (33,34). Growth medium was DMEM supplemented with 20% FBS, 1 μg/ml hydrocortisone sodium succinate, 0.2 units/ml insulin, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cell lines were routinely assessed for microbial contamination (including mycoplasma), and molecularly characterised using an inhouse panel of cellular and molecular markers to check that cell lines have not been cross contaminated (every 3-6 months; data not shown). Stocks were securely catalogued and stored, and passage numbers were strictly adhered to prevent phenotypic drift.

Supplementary tissue culture treatments. Conditioned medium (CM; 24 h) 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 72 h (CM was refreshed every 24 h). Add back experiments were carried out using 1 μM dimethyl PGE2 (Sigma-Aldrich; Merck KGaA) to the CM prior to culture.

HCA7 cells were treated with 75 μM NS-398 (Sigma-Aldrich; Merck KGaA), for 24 h, using a dose selective for COX-2 inhibition (35). Both HCA7 and HT-29 cells were treated with 100 ng/ml TNF-α (Insight Biotechnology Ltd.), and SW837 cells were treated with 10 ng/ml IL-1β (Insight Biotechnology Ltd.), both for up to 72 h. The concentration of TNF-α and IL-1β used had been previously optimized (36).

Determining percentage of floating cells as a measure of apoptosis. Growth medium from individual flasks was collected and the number of floating cells counted. Attached cell yield was determined by trypsinising 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), and used as a measure of cell death, as described previously (37).

RNA interference. Cells were transfected using Lipofectamine RNAiMAX (Invitrogen, Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, with small interfering RNAs (siRNAs final concentration 50 nM; GE Healthcare Dharmacon, Inc.) targeting BCL-3 (AGACACGCCUCUCCA UAUU) or COX-2, for which four different siRNA sequences were pooled (GGACUUAUGGGUAAUGUUA, CAUCAA CACUGCCUCAAUU, GAAAUUUGACCCAGAACUA and GAAUUACCCAGUUUGUUGA) (23). Cells were incubated overnight at 37˚C, before changing the medium. Samples were prepared up to 72 h after transfection.

Reverse transcription (RT). Total RNA was extracted from cells using TRI-reagent (Sigma-Aldrich; Merck KGaA), an RNeasy mini kit (Qiagen, Inc.) was utilised according to manufacturer's protocol with an additional on-column DNase digestion step (RNase-Free DNase Set; Qiagen, Inc.), prior to complementary (c)DNA synthesis. The RNA concentration of samples was measured using a NanoDrop (Thermo Fisher Scientific, Inc.).

cDNA was synthesised from 2 μg RNA by RT, using the RNA-dependent DNA polymerase, Moloney murine leukaemia virus reverse transcriptase, (Promega Corporation), at 40˚C for 1 h. A second tube, without reverse transcriptase was used as the negative control (no RT). The samples were diluted further giving a final concentration of 10 ng/μl.

Quantitative-PCR (qPCR). Following optimisation of primers and ensuring the annealing temperature provided ~100% amplification efficiency per cycle (data not shown), qPCR was performed as previously described (24), using SYBR Green PCR mix (Qiagen, Inc.) and the following Qiagen QuantiTect primers, at a dilution of 1:10: BCL-3, cat. no. QT00040040; and COX-2, cat. no. QT00040586; with gene expression normalised interchangeably with both housekeeping genes TATA-binding protein (TBP; cat. no. QT00000721) or Hypoxanthine Phosphoribosyl transferase (HPRT; cat. no. QT00059066). The QuantiTect primer assays sets are designed to have annealing temperatures of 55˚C. For PCR, a 40-cycle program was performed (denaturing, 15 sec at 94˚C; annealing, 30 sec at 55˚C; and extension, 30 sec at 72˚C) using a MxPro 3005P Real-time Thermal Cycler (Agilent Technologies, Inc.); samples were amplified in triplicate with one no RT well per condition. Amplification data was analysed using MxPro software version 4.10 (Agilent Technologies, Inc.), using the $2^{-\Delta\Delta Cq}$ method (38).

Immunoblotting. Whole-cell lysates were prepared *in situ,* on ice, using 100 μl 1x lysis buffer (Cell Signaling Technology, Inc.) with the addition of a Protease Inhibitor Cocktail Tablet (Roche Diagnostics) per 10 ml lysis buffer. The cell debris were removed by centrifugation at 1˚C for 10 min, at 18,500 x g. Protein concentration of the cell lysate was determined using a Bio-Rad DC Protein assay kit (Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. Absorption was measured in duplicate at 750 nm using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Inc.). Samples of 100 μg total protein were prepared in a volume of 20 μl (adjusted with distilled water), and 5 μl 5x Laemmli buffer was added to each lysate sample and boiled for 5 min.

Mini-Protean 3 Electrophoresis Cells (Bio-Rad Laboratories, Inc.) were used to cast 9% acrylamide resolving gels, using 1.5 mm spacers. Gels were run at 100 V for ~15 min, allowing the samples to move through the stacking gel, before voltage was increased to 180 V for ~1 h, 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 PVDF membrane (EMD Millipore). The gel and membrane were then assembled into a Transblot Cell (Bio-Rad Laboratories, Inc.), and a voltage of 100 V was applied for 1.5 h. The membrane was blocked in 5% (w/v) milk blocking buffer for a minimum of 1 h at room temperature, prior to immunoblotting, as previously described (39), using the following antibodies diluted in 0.5% (w/v) milk dilution buffer: Rabbit polyclonal anti-BCL-3 (1:2,000; cat. no. 23959-1-AP; ProteinTech Group, Inc.), goat polyclonal anti-COX-1 (1:500; cat. no. sc-1752; Santa Cruz Biotechnology, Inc.), goat polyclonal anti-COX-2 (1:500; cat. no. sc-1745; Santa Cruz Biotechnology, Inc.) or rabbit polyclonal anti-15-PGDH (1:2,000; cat. no. ab37148; Abcam). The membranes were incubated overnight at 4˚C, after which they were washed three times 10 min each in Tris-Buffered Saline-0.1% (v/v) Tween-20 (TBS-T). Subsequently, the membranes were incubated with an appropriate horseradish peroxidise-conjugated secondary antibody (1:1,000, anti-mouse IgG, cat. no. A4416; anti-rabbit IgG, cat. no. A6154, 1:30,000; anti-goat IgG, cat. no. A9452; all from Sigma-Aldrich; Merck KGaA) in 0.5% (w/v) milk dilution buffer. After an 1 h incubation at room temperature on a rocker, the membranes were washed three times, 10 min each, in TBS-T, and rinsed once with distilled water prior to visualisation of the signals using LumiGLO Peroxidase Chemiluminescence Substrate (Kirkegaard & Perry Laboratories, Inc.). The signal was detected using x-ray films developed in a Compact X4 Film Processor (XOgraph Imaging Systems Ltd.). The length of time films were exposed was dependent on the strength of the signal (between 1-20 min). Equal loading was confirmed using a monoclonal mouse anti-α-tubulin incubated for 2 h at room temperature (1:10,000; cat. no. T9026; Sigma-Aldrich; Merck KGaA).

Blots were quantified using ImageJ version 1.52p (National Institutes of Health). Densitometry analysis 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 Company), that used 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 an immunoassay kit (Cayman Chemical Company), as described previously (40). Cells were pre-treated with TNF-α (100 ng/ml, Insight Biotechnology Ltd.) for 16 h or NS-398 (75 μM, Sigma-Aldrich; Merck KGaA) for 24 h as required. Samples were performed in triplicate.

Statistical analysis. For statistical analysis, a one sample t-test, a Student's t-test, or a one-way ANOVA with Tukey's post-hoc test was used to compare groups in GraphPad Prism version 7 (GraphPad Software, Inc.). Results are expressed as the mean ± standard error of the mean, where a minimum of three independent experiments were performed. Where a single experiment was performed with multiple technical repeats, the data are presented as the mean of the technical repeats ± standard deviation.

Results

Knockdown of BCL-3 expression decreases COX-2 expression in colorectal cancer cells. BCL-3 consistently repressed *PTGS2* expression in a number of human colorectal cancer cell lines in focused mini-arrays (data not shown). Therefore, to determine whether BCL-3 regulated COX-2/PGE_2 signalling in colorectal cancer, HCA7 human colorectal cancer cells, which endogenously express high levels of COX-2 and high BCL-3 expression (Fig. 1A), were transfected with BCL-3 siRNA and the expression of COX-2 was determined (Fig. 1B). Cells were transfected with 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). Transfection with all four siRNA sequences separately resulted in a reduction in the levels of COX-2 protein (Fig. 1B). Sequence A was used in all subsequent experiments. The protein expression levels of both BCL-3 and COX-2 in HCA7 cells were assessed 24, 48 and 72 h post-transfection (Fig. 1C). Western blot analysis showed that the levels of COX-2 protein were

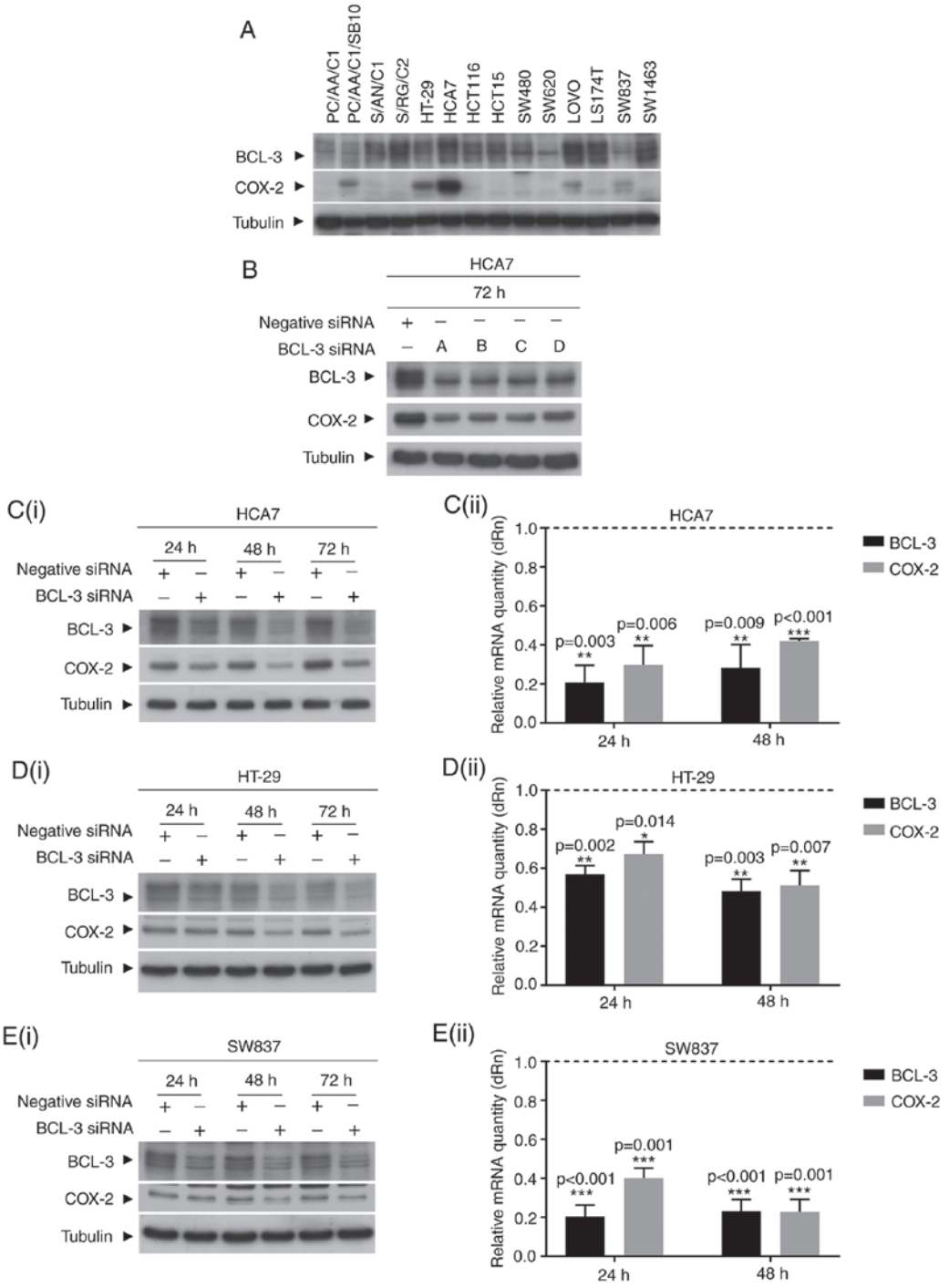

Figure 1. BCL-3 knockdown reduces COX-2 expression. (A) Endogenous levels of BCL-3 and COX-2 expression in a panel of colorectal adenoma and carcinoma derived cell lines. PC/AA/C1, S/AN/C1, S/RG/C2 colorectal cells, PC/AA/C1/SB10 transformed adenocarcinoma cells, HT-29, HCA7, HCT116, HCT15, SW480, SW620, LOVO, LS174T colorectal adenocarcinoma cells, and SW837 and SW1463 rectal adenocarcinoma cells were grown to ~70% confluence before collection of total protein for western blot analysis. α-tubulin was used as the loading control. (B) Western blot of BCL-3 expression in HCA7 cells to determine the efficiency of BCL-3 siRNA sequences. The expression levels of BCL-3 was measured in HCA7 cells 72 h after knockdown of BCL-3 using four separate BCL-3 siRNAs (A, B, C and D). The western blot is representative of two independent repeats. Western blot and RT-qPCR analysis of (C) HCA7, (D) HT-29 and (E) SW837 cells transfected with control or BCL-3 siRNA. The visible non-specific higher weight band is the result of a long exposure time used to detect low endogenous expression levels of COX-2 in the SW837 cells. i) BCL-3 siRNA transfected cells were harvested, total protein was extracted 24, 48 and 72 h post-transfection, and BCL-3 and COX-2 levels were assessed by western blotting. α-tubulin was used as the loading control. The results are representative of three independent experiments. ii) RT-qPCR was performed on cells from parallel flasks 24 and 48 h post-transfection. Relative mRNA quantity of BCL-3 and COX-2 are presented as a fold change of the control negative siRNA, which itself was normalised to one. All mRNA values were normalised to the housekeeping genes TBP or HPRT (n=4). *P≤0.05, **P≤0.01 ***P≤0.001. BCL-3, B-cell chronic lymphocytic leukaemia 3; COX-2, cyclooxygenase 2; siRNA, small interfering RNA; dRn, baseline corrected normalised fluorescence.

reduced following knockdown of BCL-3 at all-time points (Fig. 1C). BCL-3 and COX-2 mRNA levels were evaluated using RT-qPCR. Results are presented as the fold change of the negative siRNA transfected levels (Fig. 1C). Knockdown

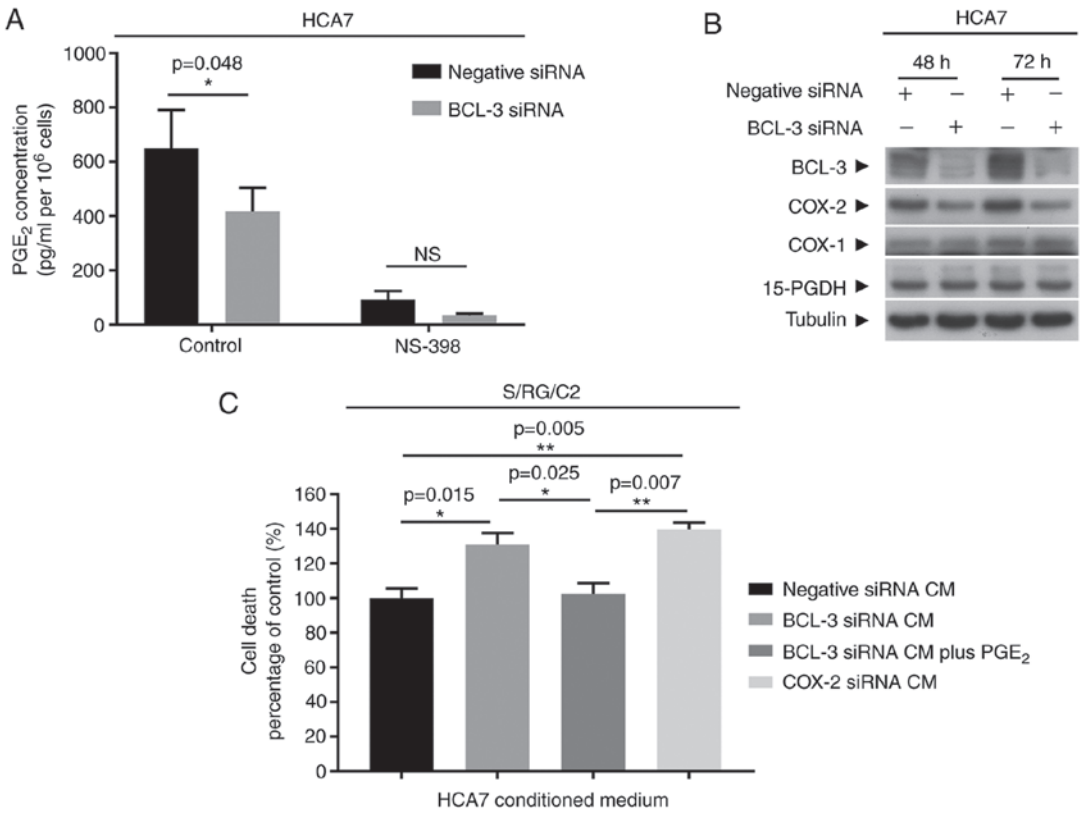

Figure 2. BCL-3 knockdown reduces PGE_2 production in the HCA7 cells. (A) Quantity of PGE_2 produced by HCA7 cells compared with NS-398 treated cells. Cells were transfected with control or BCL-3 siRNA. PGE_2 production was determined 72 h after transfection. Values are the mean of three independent experiments ± standard error of the mean. $^{*}P\leq0.05$. (B) Western blotting showed that downregulation of BCL-3 had no notable effect on COX-1 or 15-PGDH protein levels for at least up to 72 h post BCL-3 siRNA transfection. Results are representative of three independent experiments. (C) Medium exchange experiments: 24 h CM was harvested from BCL-3 siRNA, COX-2 siRNA or negative control transfected HCA7 cells and used to culture S/RG/C2 adenoma cells. Induction of cell death is represented by the number of floating cells as a proportion of total cell yield (37). Results are presented as a fold change of the control negative siRNA. PGE_2 was added to CM in the rescue experiments. Data are presented as the mean of three independent experiments ± standard error of the mean. $^{*}P\leq0.05$, $^{**}P\leq0.01$. NS, not significant; BCL-3, B-cell chronic lymphocytic leukaemia 3; COX, cyclooxygenase; siRNA, small interfering RNA; CM, conditioned medium.

of BCL-3 expression resulted in >60% reduction in COX-2 mRNA levels, confirming that knockdown of BCL-3 in colorectal cancer cells decreased both the COX-2 mRNA and protein expression levels.

To determine whether BCL-3 regulated COX-2 expression in other colorectal cancer cell lines, HT-29 (which has an intermediate level of endogenous COX-2 protein expression; Fig. 1D) and the SW837 rectal cancer cell lines (low endogenous COX-2 protein expression; Fig. 1E) were assessed. Transfection with BCL-3 siRNA also resulted in downregulation of COX-2 mRNA and protein expression levels in both cell lines.

Knockdown of BCL-3 expression decreases COX-2/PGE_2 signalling. To confirm that the decrease in COX-2 expression following transfection with BCL-3 siRNA resulted in reduced COX-2 activity, PGE_2 levels were measured in the culture medium. HCA7 cells were used for this experiment as the high endogenous COX-2 expression allowed PGE_2 to be measured directly by ELISA (Fig. 2A). COX-1 and 15-PGDH protein expression were also assessed by western blotting 72 h after BCL-3 siRNA transfection (Fig. 2B) to determine whether expression of any other enzymes which regulate PGE_2 levels were altered. Furthermore, cells were treated with NS-398 to validate that changes in PGE_2 detected in the cell culture medium were due to COX-2 and not COX-1 activity (Fig. 2A). The results showed that PGE_2 production was significantly reduced when BCL-3 expression was knocked down (P=0.048); there was a ~30% decrease in basal PGE_2 production in BCL-3 siRNA transfected cells compared with the negative siRNA control. The levels of PGE_2 were reduced by the COX-2 selective inhibitor NS-398 by >90% (Fig. 2A). Finally, COX-1 and 15-PGDH levels were not altered by transfection with the BCL-3 siRNA (Fig. 2B). Taken together, these findings show that the observed decrease in PGE_2 production in the BCL-3 siRNA transfected cells was due to a loss of COX-2 activity.

Subsequently, whether the regulation of PGE_2 observed by suppression of BCL-3 affected survival of other tumour cells was determined. CM exchange experiments were performed using a PGE_2 sensitive adenoma derived cell line (S/RG/C2). It was previously shown that PGE_2 promoted the growth of S/RG/C2 (41). CM from the BCL-3 siRNA, COX-2 siRNA or negative control transfected HCA7 cells were harvested and used to culture the S/RG/C2 adenoma derived cell lines. The number of HCA7 cells were not affected by BCL-3 knockdown or COX-2 downregulation during the preparation of CM over the 72 h period, (Fig. S1A). Treating S/RG/C2 cells with CM from the BCL-3 siRNA transfected HCA7 cells

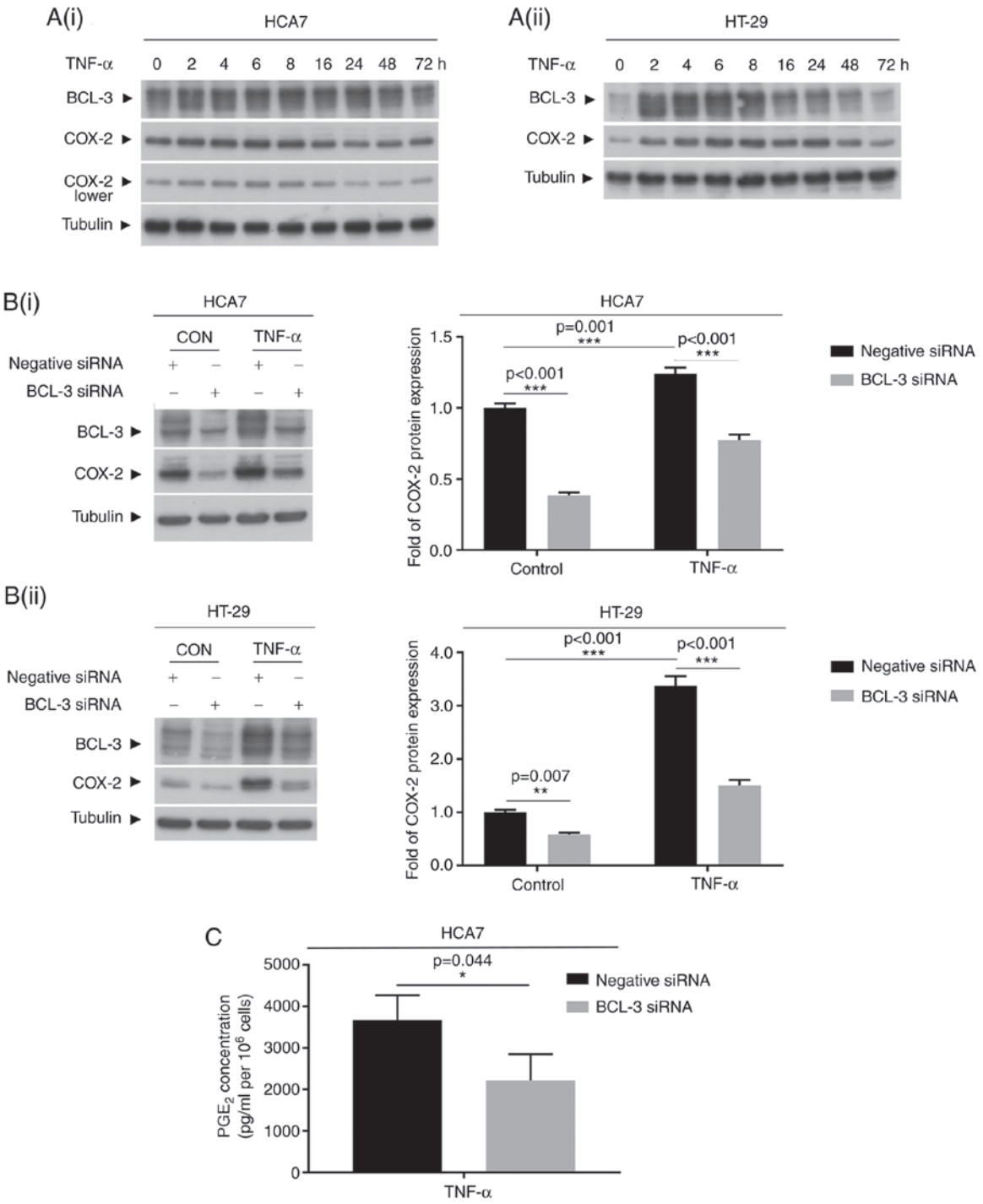

Figure 3. TNF-α induced increases in COX-2 expression is partially blocked following knockdown of BCL-3 in siRNA treated cells. (A) Western blotting shows the expression of BCL-3 and COX-2 proteins following TNF-α treatment in i) HCA7 and ii) HT-29 cells. The results are representative of three independent experiments. (B) Representative western blot showing the expression of COX-2 protein following the knockdown of BCL-3 in TNF-α treated cells. i) HCA7 and ii) HT-29 cells were transfected with control or BCL-3 siRNA and treated with TNF-α for 4 h. BCL-3 and COX-2 protein expression levels were determined 72 h post-transfection. Equal loading was confirmed by α-tubulin. Graphs show the fold change of COX-2 protein expression following TNF-α treatment. Data are presented as the mean ± standard deviation of three independent repeats. $^{**}P\leq0.01$ and $^{***}P\leq0.001$ (C) Quantity of PGE_2 produced in HCA7 cells treated with TNF-α. HCA7 cells were transfected with control or BCL-3 siRNA. PGE_2 production was determined 72 h after siRNA transfection. Values are presented as the mean ± standard error of the mean of three independent repeats. $^{*}P\leq0.05$. TNF-α, tumour necrosis factor-α; COX-2, cyclooxygenase-2; BCL-3, B-cell chronic lymphocytic leukaemia 3; small interfering RNA; PGE_2, prostaglandin E2; CON, control.

resulted in a significant increase in floating cells (P=0.015). Cell death was rescued by addition of dimethyl PGE_2 to the CM prior to culture. In addition, the degree of cell death was similar to that observed in cells grown in CM from HCA7 cells transfected with the COX-2 siRNA (Fig. 2C). These findings suggest that the changes in PGE_2 secretion caused by knockdown of BCL-3 affected the survival of PGE_2 sensitive tumour cells.

Together these results showed that knockdown of BCL-3 expression downregulated expression of COX-2

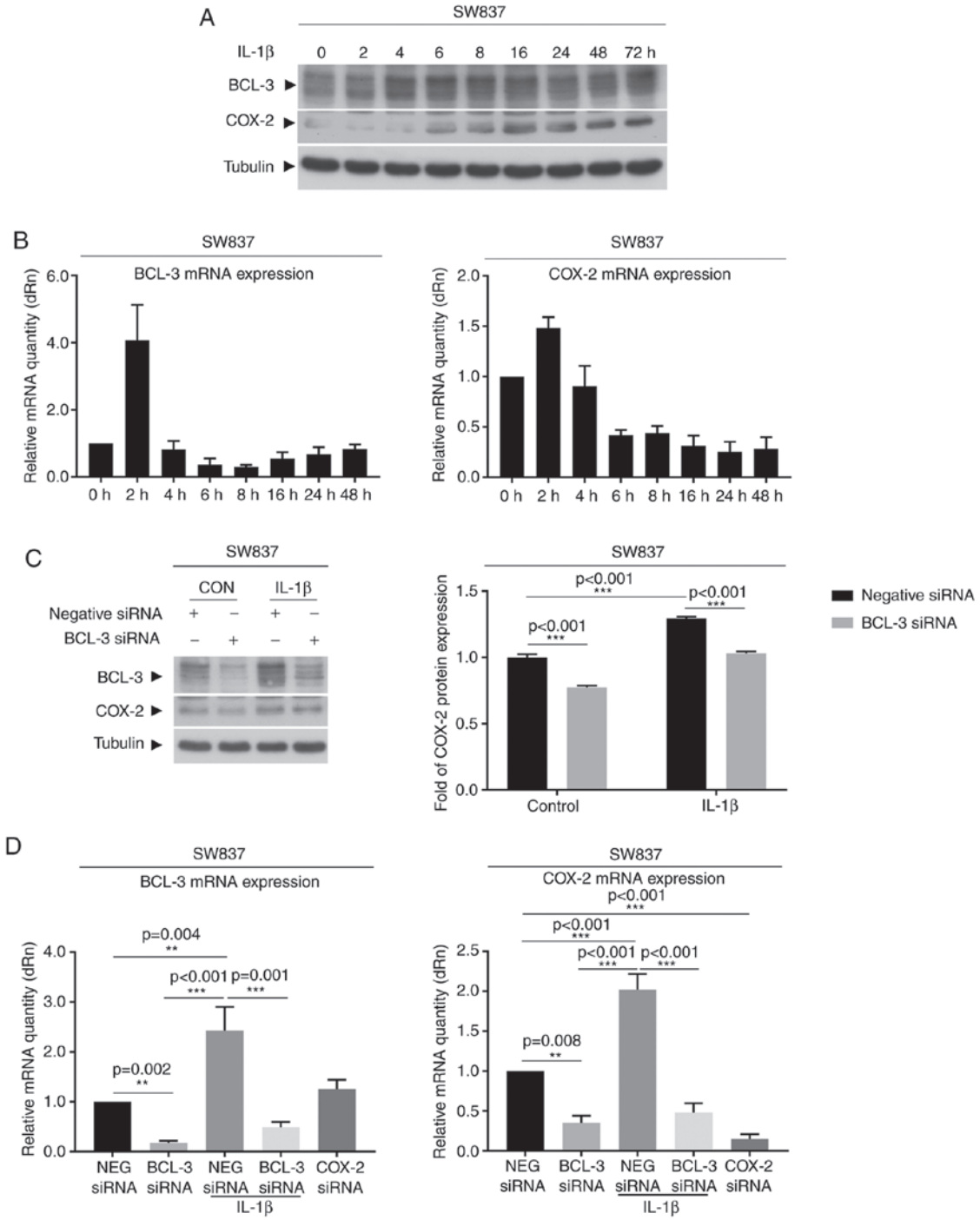

Figure 4. IL-1β induced increase in COX-2 protein expression is partially blocked following knockdown of BCL-3 in SW837 cells. (A) Western blots showing BCL-3 and COX-2 protein expression following IL-1β treatment. Results are representative of three independent repeats. (B) RT-qPCR was performed on cells treated with IL-1β. Data are presented as a fold change of the untreated control. All mRNA values were normalised to the housekeeping gene TBP or HPRT. n=4. (C) Representative western blots showing the expression of COX-2 protein following knockdown of BCL-3 in the IL-1β treated cells. SW837 cells were transfected with control or BCL-3 siRNA and treated with IL-1β for 4 h. BCL-3 and COX-2 protein levels were determined 72 h post-transfection. α-tubulin was used as the loading control. The graph shows the fold change of COX-2 protein expression following IL-1β treatment. Expression is normalized to the respective loading control. Data are presented as the mean ± standard deviation. n=3. ***P≤0.001. (D) BCL-3 and COX-2 mRNA expression levels following transfection with BCL-3 or COX-2 siRNA. RT-qPCR was performed following treatment with IL-1β for 2, 72 h post-transfection. Results are presented as the fold change of the control negative siRNA. All mRNA values were normalised to the housekeeping gene TBP or HPRT. There was a significant reduction in COX-2 mRNA levels in cells transfected with BCL-3 siRNA, including after IL-1β treatment for 2 h. Transfection with COX-2 siRNA had no effect on the levels of BCL-3 expression. Data are presented as the mean ± standard error of the mean. n=4. **P≤0.01, ***P≤0.001. IL, interleukin; COX-2, cyclooxygenase-2; BCL-3, B-cell chronic lymphocytic leukaemia 3; small interfering RNA; RT-qPCR, reverse transcription-quantitative; CON, control; dRn, baseline corrected normalised fluorescence.

and significantly reduced the quantity of potentially pro-tumorigenic PGE_2 produced by the cancer cells.

Induction of COX-2 expression by TNF-α or IL-1β is partially blocked by knockdown of BCL-3. 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 (31). These cytokines have also been shown to increase COX-2 expression in colorectal cancer cells (4,42,43). To determine whether targeting BCL-3 reduced the induction of COX-2 in response to inflammatory cytokines, HCA7 and HT-29 cells were treated with 100 ng/ml TNF-α for up to 72 h (Fig. 3), and SW837 were treated with 10 ng/ml IL-1β (Fig. 4) as SW837 cells are more responsive to IL-1β compared with TNF-α. BCL-3 and COX-2 protein expression were measured over 72 h. BCL-3 protein levels increased after 2 h of treatment with TNF-α (Fig. 3A), reaching peak levels after 4-8 h of treatment and returning to basal levels by 72 h. COX-2 expression followed a similar pattern to BCL-3 induction; COX-2 was induced after 4-8 h, and remained high for 24-48 h when treated with TNF-α, consistent with its regulation with BCL-3 (Fig. 3A). Notably, the increased expression of COX-2 in response to TNF-α treatment (as shown in Fig. 3B at 4 h) was found to be partially blocked following the knockdown of BCL-3 in both HCA7 and HT-29 cell lines. Suppression of BCL-3 also reduced PGE_2 production by the HCA7 cells in response to TNF-α treatment (Fig. 3C). Similar results were observed in the SW837 cells treated with 10 ng/ml IL-1β (Fig.4). Due to the low endogenous levels of COX-2 protein expression in the SW837 cells, expression of both BCL-3 and COX-2 were also measured using RT-qPCR (Fig. 4B and D). There was a rapid induction of BCL-3 (within 2 h) following treatment with IL-1β, followed by a more prolonged induction of COX-2 (Fig. 4A and B). In addition, the increase in COX-2 expression 2-4 h after IL-1β treatment was partially blocked following knockdown of BCL-3 (Fig. 4C and D). These data show that inhibition of BCL-3 expression not only reduced basal COX-2 expression levels but also significantly supressed cytokine induction mediated by COX-2. These results suggest that expression of BCL-3 may be an important determinant in the COX-2-mediated response to inflammatory cytokines.

Discussion

Discovered as an oncogene in leukaemia, BCL-3 has been shown to be overexpressed in a range of solid tumours including breast (21), prostate (44), endometrial (45) and nasopharyngeal carcinoma (46). The importance of BCL-3 in colorectal carcinogenesis is supported by both clinical and mechanistic studies. Puvvada *et al* (26) were the first to show that high levels of nuclear BCL-3 were correlated with a poor prognosis in patients with colorectal cancer, and Saamarthy *et al* (27) proposed BCL-3 cellular localisation as a marker for early diagnosis in colorectal cancer. Both studies reported that at least 30% of colorectal tumours had increased nuclear expression of BCL-3 (26,27). More recently high BCL-3 expression has been shown to be associated with worse survival in CRC (24).

There are several studies which have begun to elucidate the mechanism by which BCL-3 promotes colorectal carcinogenesis; in our previous study, it was shown that BCL-3 expression promotes AKT mediated cell survival and drives colorectal tumour growth *in vivo* (23), and BCL-3 has also been reported to increase the stability of c-MYC via activation of ERK (25). In addition, BCL-3 has been implicated in promoting tumorigenesis through inhibition of DNA damage induced p53 activation by upregulating MDM2 expression (47), and in inducing cyclin D activity, and thus, cell cycle progression (48). More recently, it was shown that BCL-3 promoted development of a cancer stem cell phenotype by enhancing β-catenin signalling in colorectal tumour cells (24).

Given the importance of upregulated COX-2 expression in colorectal tumorigenesis, the novel finding that BCL-3 regulates *PTGS2* gene expression and COX-2/PGE_2 signalling, highlights a new mechanism underlying the oncogenic actions of BCL-3 in colorectal cancer. Although not required for COX-2 expression, it may be hypothesized that nuclear BCL-3 serves as a transcriptional co-factor, as recently proposed for β-catenin-dependent gene targets (24). It remains to be determined whether BCL-3 enhances NF-κB-dependent COX-2 regulation or acts via recruitment of other co-regulators, such as Pirin, Tip60, Jab1 and Bard1 (49). The ability of BCL-3 to regulate PGE_2 production is of particular importance; not only does PGE_2 promote expression of the intestinal cancer stem cell marker LGR5 (41), but PGE_2 production by colorectal cancer cells, as well as by melanoma and breast cancer cells, suppresses tumour immunity and drives tumour promoting inflammation (50). COX-dependent immune evasion has been shown to be critical for tumour growth in colorectal cancer mouse models, as COX-2 has been reported to be responsible for the majority of circulating PGE_2 (31,50). A limitation of the present study is that TNF-α and IL-1β are only two of the upstream regulators of COX-2 activity, whether BCL-3 expression regulates the response to other factors remains to be determined. Furthermore, the potential effects of the identified mechanism on the numerous downstream targets of COX-2/PGE_2 signalling remains to be determined (6).

In the present study, it was shown that targeting BCL-3 expression may suppress COX-2/PGE_2 signalling, which not only impacts the growth of other tumour cells, but may also potentially enhance the immune response to the cancer.

Taken together, and given the importance of these pathways in tumour progression, targeting BCL-3 protein-protein interactions (via disruption of the ankyrin repeat domain for example) remains a priority. Inhibitors of the BCL-3 pathway currently under development (Dr Richard Clarkson, Cardiff University) may 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 that BCL-3 inhibitors may be used as an alternative or complementary approach to using NSAIDs for prevention and/or preventing recurrence in PGE_2-driven tumorigenesis (51), and may improve long-term prognosis for patients with colorectal cancer.

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Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ACW, HF, TJC, CP and AG conceived and designed the study. ACW, HF, TJC and AG developed the methodology. ACW, HF, TJC, CP and AG acquired the data. ACW, HF, TJC, CP and AG analysed and interpreted the data. TJC and ACW wrote, reviewed and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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