



Original Articles

An old spice with new tricks: Curcumin targets adenoma and colorectal cancer stem-like cells associated with poor survival outcomes

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ABSTRACT

The cost of cancer care globally is unsustainable and strategies to reduce the mounting burden of cancer are urgently needed. One approach is the use of preventive therapies to reduce cancer risk; dietary-derived compounds with good safety profiles represent a promising source of potential candidates but translating encouraging preclinical data to successful trials presents significant challenges. Development of curcumin, from the spice turmeric, as a preventive therapy for colorectal cancer (CRC) is hindered by poor understanding of its mechanism of action. Using patient derived xenografts and *ex-vivo* 3D-models exposed to clinically achievable curcumin concentrations, we found that it targets proliferating cancer stem-like cells (CSCs) within premalignant adenoma and early-stage cancer tissues, with broad spectrum activity across all molecular subtypes. Transcriptomics analysis revealed that curcumin pushes CSCs towards differentiation over self-renewal, thereby inhibiting tumour development. Evidence suggests these effects involve direct protein binding of curcumin to NANOG, a master regulator of CRC CSCs, and impairment of its transcriptional activity via direct interference with NANOG-DNA binding. Furthermore, curcumin decreased the proportion of proliferating CSCs, defined by NANOG/Ki67 co-expression in patient derived explants and individuals with tumours containing a small fraction of these cells had greatly improved progression-free survival compared to those in the highest quartile for expression. The use of curcumin to minimise this cellular population may yield significant benefit and its clinical evaluation is warranted. Overall, this study provides crucial mechanistic insight, identifying patient populations

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likely to benefit from curcumin for prevention of sporadic CRC and theragnostic biomarkers for assessing efficacy.

1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths worldwide [1]. Whilst earlier detection via screening programmes and improved treatment options has led to a decline in mortality in industrialised countries, mortality rates are rising in developing nations, likely related to an increasingly westernised lifestyle and late detection [1]. Additionally, increased CRC incidence has been observed in young individuals (aged 25–49 years), who are often diagnosed at an advanced stage, resulting in greater overall loss of life years [2]. As the increasing burden and mounting costs of cancer care are not sustainable, greater prominence must be given to the development of preventive strategies to reduce cancer incidence [3].

Therapeutic cancer prevention offers an innovative approach to help reduce the burden of CRC; the progression from adenoma to CRC has a long latency period of years to decades, through a multistep process with accumulation of mutations [4]. This provides opportunity for therapeutic interventions that may block or intercept CRC development [5]. As early neoplasia are characterised by fewer genetic alterations and less tumour heterogeneity compared with advanced malignancies, there is a strong rationale for targeting pre- or early malignant cells, with an increased potential for successful eradication [6]. In the context of sporadic CRC, the use of aspirin for prevention is supported by epidemiological and randomised controlled trial (RCT) data [7]. However, aspirin will not work for everyone and may be contraindicated in others; therefore, there is a need to increase the options for cancer prevention to enable a precision prevention approach. Dietary-derived agents that offer a favourable safety profile have long been a focus of efforts to identify novel cancer preventive therapies. To effectively develop such therapies it is important to elucidate the key mechanisms of action; this is crucial for developing companion biomarkers that can be used to identify patients likely to benefit from therapy and allow efficacy monitoring [3].

Tumours exhibit substantial intra-tumour heterogeneity and cellular plasticity, supporting clonal evolution of cancer cells [8]. Additionally, a group of cells, known as cancer stem-like cells (CSCs) harbour characteristics that make them more likely to initiate cancer development, drive metastasis and resistance to standard therapies, compared to differentiated tumour cells [8]. This has generated pharmaceutical interest in development of novel agents and repurposed drugs to specifically target CSCs in solid tumours [9]. In contrast, there has been little focus on this approach for cancer prevention to date.

CSCs are regulated by three key pluripotency genes; *POU5F1*, which encodes Octamer-binding transcription factor-4 (OCT4), Sex determining region Y (*SOX2*), and *NANOG*, which is considered the master regulator of CRC CSCs [10]. Plasticity of CSCs has been proposed, where cells possess the capacity to move between a stem-like and differentiated state within a tumour based on their internal or external environment [8]. This suggests that for effective cancer interception, multiple pathways should be targeted using a combination of therapies or a single agent that modulates multiple pathways.

Curcumin is a bright yellow polyphenol found in the spice turmeric and is widely used as a dietary supplement. It has low systemic bioavailability but provides pharmacologically active concentrations in colonic tissues, rendering it a good candidate for CRC management [11]. Curcumin modulates multiple pathways which contribute to the development of CRC, it has low toxicity and is inexpensive [12], satisfying many of the requirements of an ideal preventive therapy. A number of RCTs support curcumin use in cancer prevention, however trials to date have been relatively small and provided inadequate evidence on key molecular targets that could potentially serve as pharmacodynamic

biomarkers [13].

In this study, we address one of the main barriers to advancing curcumin as a therapy for CRC prevention; identifying key mechanisms of action. Curcumin was found to target proliferating CSCs within pre-malignant adenoma and early-stage cancer tissues, pushing them towards differentiation, thereby inhibiting tumour development and growth. We propose this process involves direct-binding of curcumin to the NANOG protein, and impairment of its transcriptional activity via direct interference with NANOG-DNA binding. We show that patients with tumours containing the lowest fractions of proliferating CSCs, characterised by NANOG/Ki67 co-expression, present with greatly improved progression-free survival compared to those in the highest quartile, suggesting that use of curcumin to reduce this cell population has potential to lead to significant clinical benefit.

2. Results

2.1. Curcumin demonstrates activity against CSCs in human ex-vivo spheroids

We profiled 10 adenomas, 50 cancers, 6 colorectal liver metastases and 45 samples of normal colorectal tissue obtained from patients undergoing surgical resection to verify the relevance of targeting CSCs within adenomas as a strategy for CRC prevention (Fig. 1A–B and Supplemental Table S1A). CSCs were defined using fluorescence activated cell sorting (FACS) analysis based on ALDH activity and CD133 expression, combined with expression of epithelial surface antigen (ESA⁺). A larger population of ALDH1A1-expressing cells in adenomas has been associated with a higher risk for subsequent metachronous adenoma, and CD133 is a well-established marker for cancer cells with a CSC phenotype but less is known about its role in premalignant tissue [14,15]. The proportion of ALDH^{high}/CD133⁺ cells was significantly higher in adenomas (11.8 %) compared to normal tissue (4.8 %) ($p < 0.001$), and all cancers combined (2.9 %) ($p < 0.01$). CD133⁺ALDH^{low} cells were present at highest levels in cancer tissues (12.1 %), with a 10-fold lower fraction in adenoma and normal tissue ($p < 0.0001$) (Fig. 1A and B). ALDH^{high}/CD133⁺ cells represented a minor proportion of cells but were significantly higher in cancers (0.80 %) compared to adenomas (0.62 %) and normal tissues (0.21 %) ($p < 0.001$). Overall, these findings support targeting cells with ALDH^{high} activity, particularly the ALDH^{high}CD133⁺ population in adenomas for CRC prevention.

Using clinically achievable concentrations [11], we demonstrated the ability of curcumin to inhibit growth and expansion of patient-derived CSCs from CRC and adenoma (Fig. 1C–E). Cells were seeded in curcumin-containing medium with fresh medium added twice weekly for 4 weeks to mimic repeat dosing in a clinical situation. A significant reduction in sphere number and proportion of ALDH^{high} cells was observed, but there were differing dose responses among patients and a larger effect on sphere number compared to size (Fig. 1C–E and Supplemental Fig. S1). In selected samples, a higher concentration of curcumin (10 μ M) was also employed, reducing sphere formation to ~5 % of control in two out of three patients. Analysis of key-driver mutations revealed spheroid inhibition in samples bearing kirsten rat sarcoma virus (*KRAS*) and proto-oncogene B-Raf (*BRAF*) mutations, suggesting that curcumin has broad activity across adenomas and CRC (Supplemental Table S2). There was a significant positive correlation between the sphere-forming ability of samples, scored on a scale of 1–4, and ALDH activity ($R^2 = 0.57$, $p < 0.001$), but such an association was not evident for CD133⁺ expression (Fig. 1F). This reinforces the premise that cells with ALDH^{high} activity are a suitable target for curcumin-mediated CRC prevention.

2.2. Curcumin inhibits tumour development in a patient-derived xenograft model generated from ALDH^{high} cells

The CSC-targeting activity of curcumin translated to *in-vivo* efficacy when human CSCs cells, isolated by FACS on the basis of ALDH^{high} activity, were transplanted subcutaneously into NOD/SCID mice. Curcumin, given as a phytosomal formulation (Meriva® 1.13 %, equivalent to 0.2 % curcumin) mixed with standard diet, significantly delayed tumour development, as shown by an increased time to first tumour palpability from 80 days in control animals to 105 days in animals receiving curcumin ($p < 0.05$) (Fig. 2A). Curcumin significantly improved survival of mice, from a median of 117 days in the control group to 160 days in the curcumin group ($p < 0.05$) (Fig. 2B). Curcumin also significantly reduced the CSC population *in vivo*. The ALDH^{high}/ESA⁺ and CD133⁺/ESA⁺ fractions were reduced by greater than 50 %, whilst the ALDH^{high}/CD133⁺ population was reduced by 83 % compared to control mice (Fig. 2C).

It is well established that curcumin has poor bioavailability, but appreciable concentrations can be detected in gastrointestinal tissue when it is taken orally in its standard form [11]. Since this xenograft study involved subcutaneous injection of cells into the flanks of mice, delivery of curcumin and its metabolites was reliant on the systemic circulation rather than any direct mucosal contact. Consequently, we opted for a phytosomal formulation that generates ~4-fold higher plasma concentrations of total curcuminoids compared to the standard curcumin [16] used in our previous study in *Apc*^{Min/+} mice [17]; these mice represent a model of hereditary-CRC characterized by a mutation in the adenomatous polyposis coli (*Apc*) gene, which leads to multiple intestinal adenomas. Moreover, the choice of dose for the xenograft study (equivalent to 0.2 % curcumin) was informed by our previous results in the *Apc*^{Min/+} mice, since we demonstrated that dietary doses of standard curcumin (0.2 and 0.5 %) reduced adenoma number by ~40 % in these animals [17].

To further investigate the ability of curcumin to target the stem components during early carcinogenesis, doublecortin CAM kinase-like 1 (DCAMKL-1) was assessed in archival intestine tissue from the

Apc^{Min/+} study. DCAMKL-1, also known as DCLK1, is a gastrointestinal tuft cell marker that identifies quiescent and tumour-growth sustaining stem cells that are able to initiate CRC [18]. DCAMKL-1⁺ cells are enriched within intestinal tissues of *Apc*^{Min/+} mice compared to wild type animals and have been used as a marker to assess the efficacy of polyp preventive agents including celecoxib [19]. It has been suggested that a subset of DCAMKL-1 expressing CSCs may be resistant to curcumin upon *in vitro* culture of spheroids derived from HCT116 colorectal cancer cell lines and treatment of athymic SCID/nude mice harbouring established tumours originating from the same cell line [20]. In our model, curcumin significantly decreased the number of cells expressing DCAMKL-1 in villi and crypts by up to ~80 % relative to control (Fig. 2D–F). To examine the effects of curcumin on normal intestinal stem-cells, wild-type mice were administered dietary curcumin following an identical 15-week protocol to our *Apc*^{Min/+} mouse study. Curcumin had no effect on the number of DCAMKL-1⁺ cells in wild-type animals, suggesting it selectively targets CSCs whilst sparing the normal intestine (Fig. 2E).

2.3. Curcumin binds to the cancer stem-cell regulating protein NANOG, interferes with NANOG-DNA binding and inhibits transcriptional activity

To investigate mechanisms underlying the efficacy of curcumin, we examined its effect on NANOG [10]. Analysis of NANOG protein expression by Western blotting in tissues from patients showed high levels in cancers and adenomas with minimal expression in adjacent matched-normal tissues (Fig. 3A–B). Additionally, the proportion of NANOG-expressing cells measured by FACS was ~8-fold higher in adenomas compared to normal tissues ($p < 0.001$) across a panel of patient samples. Furthermore, NANOG-expressing cells were significantly higher in all stages of cancer compared to normal tissues (Fig. 3C–Supplemental Table S1B). The expression profile of NANOG mirrored the pattern of ALDH activity, with the highest proportion of NANOG-expressing cells in adenomas compared to any cancer stage (Figs. 3C and 1B). These findings are consistent with previous reports suggesting NANOG is readily measurable in CRC samples but is

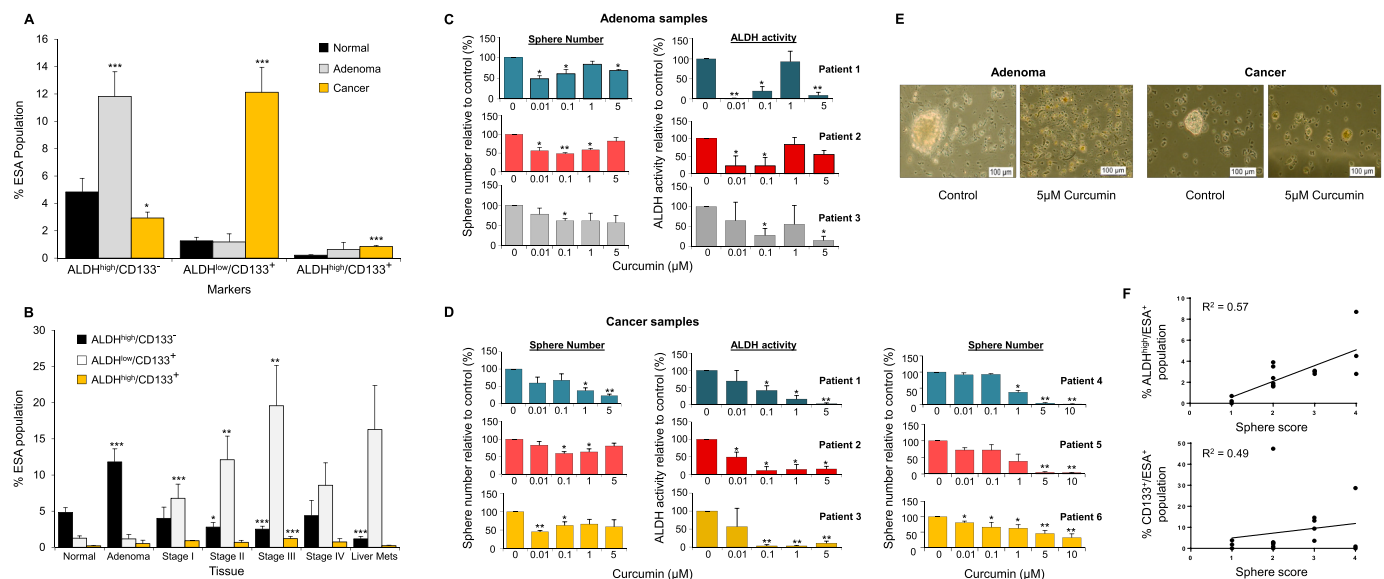


Fig. 1. Curcumin demonstrates activity against CSCs in a human *ex-vivo* spheroid model of colorectal tissues. Normal colorectal tissue, adenoma and CRCs were profiled for ALDH activity and CD133 expression and populations were quantified across these tissue types (A) and relative to cancer stage (B). (C–E) Single cells from three adenoma and six cancer tissues were plated with curcumin-containing sphere medium or vehicle (0.01–5 μ M) twice weekly for four weeks. Effects on sphere number and ALDH activity are shown for each individual patient, with cancer patients 4–6 including an additional high concentration (10 μ M). For each sample, triplicate experiments were performed on three separate occasions. Values represent mean $\pm$ SEM and significant differences relative to solvent control are shown, where * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. (F) Relationship between CSC markers employed and sphere forming ability of untreated cells (from $n = 16$ cancer and 6 adenoma samples), based on the time taken to form spheres (defined as $\geq 30 \mu$ m in size). A score of 1 = > 4 weeks, 2 = 3–4 weeks, 3 = 2–3 weeks, and 4 = < 2 weeks. Regression analysis was performed; correlations are shown by R^2 values.

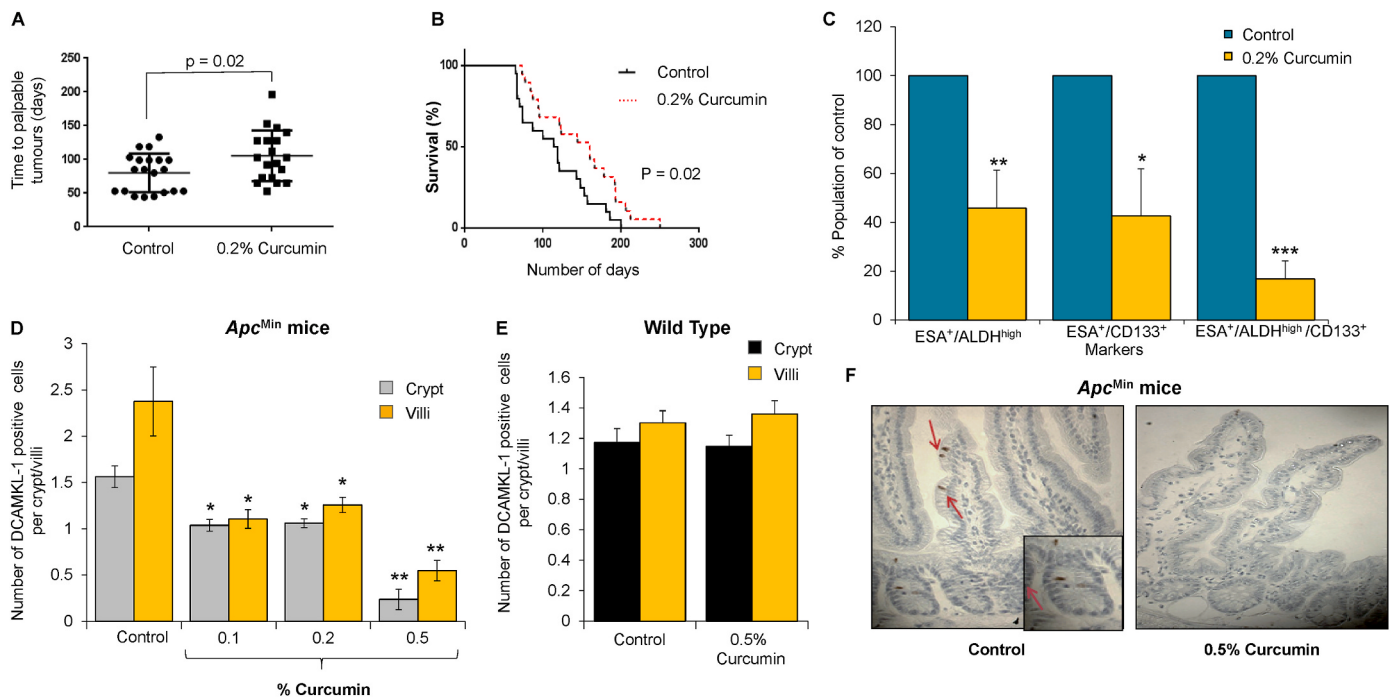


Fig. 2. Curcumin delays tumour development in a primary CRC ALDH^{high} xenograft model. NOD/SCID mice received diet containing Meriva (n = 20) (1.13 % equivalent to 0.2 % curcumin) or 0.9 % Epikuron, the control phytosomal delivery system (n = 19), for one week prior to subcutaneous injection of 2000 ALDH^{high} cells that originated from a single CRC patient. **(A)** Curcumin significantly delayed tumour development, assessed by average time to first tumour palpability (p < 0.05). **(B)** The survival of mice was extended from a median of 117–160 days in the control versus curcumin group (p < 0.05). **(C)** Curcumin decreased the proportion of ALDH^{high}/ESA⁺, CD133⁺/ESA⁺ and ALDH^{high}/CD133⁺ cells in tumours of treated mice compared to controls. Data are the mean + SEM and significant differences are shown, where * indicates p < 0.05, **p < 0.01 and ***p < 0.001. **(D–F)** Male *Apc*^{Min/+} or wild-type mice (n = 5 per group) were fed standard curcumin (0.1, 0.2, 0.5 %) or control diet from weaning until 16 weeks of age. DCAMKL-1 expression was determined by immunohistochemistry analysis of intestinal tissue, with ~30–40 villi and crypts counted per section. Values represent the mean ± SEM, with significant differences between the proportion of DCAMKL-1⁺ cells in the crypts/villi of treated and control groups indicated, where * represents p < 0.05, **p < 0.01.

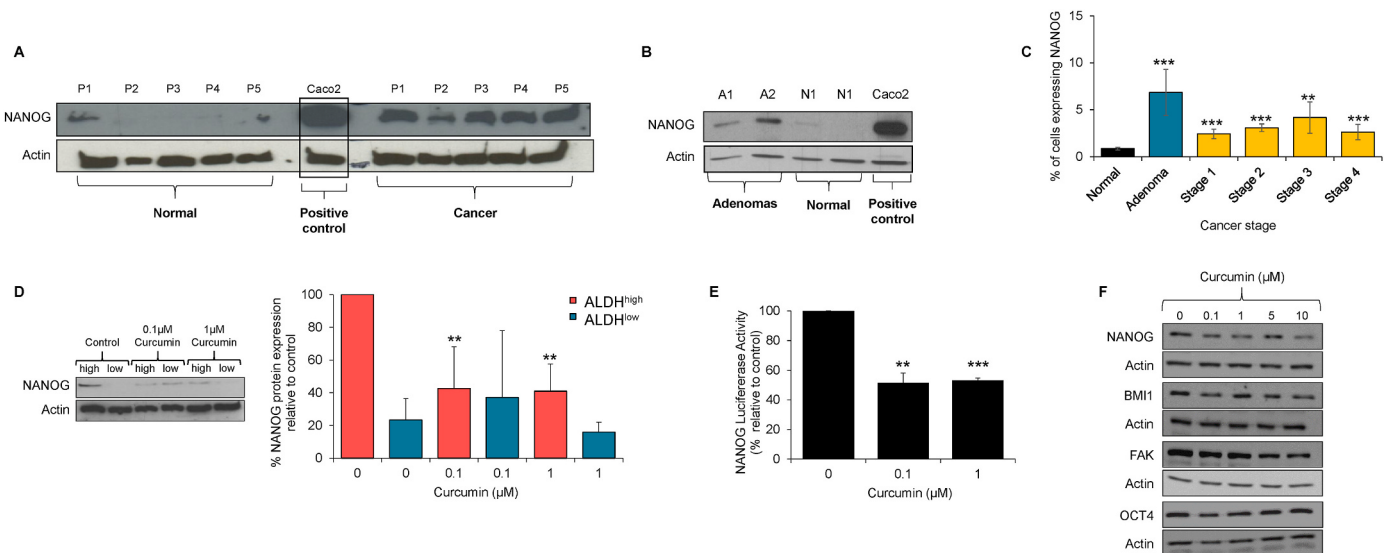


Fig. 3. NANOG is selectively expressed in colorectal cancer and adenoma tissues of patients, and curcumin reduces its expression and inhibits transcriptional activity. Western blot analysis of NANOG expression in cancer **(A)**, adenoma **(B)** and adjacent normal tissue from patients **(A–B)**; Caco2 cell lysate was a positive control. **(C)** FACS-based quantitation of NANOG expressing cells (mean ± SEM) in 6 human adenoma, 46 CRC and 38 adjacent normal tissue samples. Significant differences are shown, ** indicates p < 0.01, ***p < 0.001. **(D)** Caco2 cells were treated with curcumin on alternate days for one week. Cells were FACS-isolated based on ALDH activity and NANOG expression assessed by Western blotting. Three independent biological replicates were performed. Values represent the mean + SEM. Significant differences relative to ALDH^{high} or ALDH^{low} solvent treated cells are shown, where ** indicates p < 0.01. **(E)** Analysis of endogenous NANOG transcriptional activity in Caco2 cells incubated with curcumin for 24 h using a luciferase-reporter assay. Values represent mean + SEM of 6 independent experiments; significant differences relative to solvent control are shown, ** indicates p < 0.01, ***p < 0.001. **(F)** Western blot analysis of NANOG and its downstream targets in Caco2 cells exposed to 0–10 μM curcumin for 24 h. Three biological replicates were performed on different occasions.

non-detectable, or at very low levels, in normal tissues [21,22]. Higher NANOG-expression has been associated with later Dukes staging and poor prognosis [22]. Although less is known about premalignant tissue, it has been suggested that higher levels of CSC related genes, including *NANOG*, are expressed in villous adenoma and high-grade dysplastic cells, as well as precancerous lesions in cervix, gastric and laryngeal tissues [21], making it a candidate for targeting with preventive therapies.

To investigate how curcumin regulates NANOG in CRC, the Caco2 human colorectal adenocarcinoma cell line was used. The isolated $ALDH^{\text{high}}$ fraction of Caco2 cells had greater sphere forming ability than $ALDH^{\text{low}}$ cells, indicative of a stem-like phenotype (Supplemental Fig. S2A). Moreover, whilst curcumin was able to inhibit growth of spheres generated from the bulk Caco2 cell population (Supplemental Fig. S2B), consistent with its activity in primary-cells, the effect was limited to the stem-like $ALDH^{\text{high}}$ fraction in which it also reduced NANOG protein expression (Fig. 3D and Supplemental Fig. S2A); it had no effect on sphere formation by $ALDH^{\text{low}}$ cells (Supplemental Fig. S2A).

Using a luciferase-reporter assay and unsorted Caco2 cells cultured with low concentrations of curcumin (0.1 and 1 μM) for 24 h, a 50 % reduction in transcriptional activity of endogenous NANOG was observed (Fig. 3E), with a smaller reduction in NANOG protein expression of ~25 % (Fig. 3F and Supplemental Fig. S3A). To assess downstream ramifications, the direct targets of NANOG, focal adhesion kinase (FAK) and BMI1 proto-oncogene polycomb ring finger (*BMI1*), were evaluated [23,24]. In Caco2 cells, curcumin treatment led to significant reductions in FAK and BMI1 protein levels; in contrast, although there was an overall decrease in the levels of NANOG, there were no changes in the proportion of phosphorylated NANOG-pSerine71 (S71), OCT4 protein or NANOG mRNA expression (Fig. 3F and Supplemental Fig. S3A–G). It is important to note that phosphorylation of NANOG is essential in regulating BMI1 transcription, but that this is dependent on phosphorylation of NANOG residues Threonine 200 and Threonine 280 by protein kinase C ϵ [24], and there is currently a lack of available antibodies to detect phosphorylation at these sites. These observations therefore suggest that curcumin interferes with transcriptional activity, due to either reduced total NANOG protein levels or direct interference, independent of S71 phosphorylation.

To investigate whether curcumin binds to NANOG protein we conducted an affinity pull-down assay with novel curcumin-coupled beads synthesized in-house (Supplemental Fig. S4). The beads, which contain curcumin-coupled to an agarose resin via a 13-atom-linker, selectively

and reproducibly pulled NANOG out of Caco2 cell lysates, as evidenced by a lack of detectable binding to ethanolamine-capped control beads bearing the same linker (Fig. 4A). To ascertain whether this was direct binding of NANOG to curcumin as opposed to indirect binding through a larger NANOG-protein complex, we conducted assays using a commercially available recombinant protein and similarly demonstrated this specific interaction (Fig. 4A). To interrogate the site of curcumin binding to NANOG, we synthesized flag3-tagged truncated proteins, plus the full-length protein for use in pull-down assays. We confirmed direct binding to the full-length protein, the homeodomain and C-terminus (Fig. 4B). Using electrophoretic mobility shift assays (EMSA), curcumin binding to NANOG and the homeobox was shown to interfere with these proteins binding to DNA containing a known binding motif in a concentration-dependent manner, suggesting curcumin impairs transcriptional activity via direct interference with NANOG-DNA binding (Fig. 4C). Taken together, these data demonstrate that curcumin binds NANOG protein, interferes with NANOG-DNA binding, and reduces NANOG mediated transcription, resulting in a diminished cell population harbouring stem-like features.

2.4. Curcumin preferentially targets NANOG over-expressing cells

As *NANOG* knock-down has been shown to completely prevent sphere growth from prostate cancer cells [25] we compared the effect of curcumin in HCT116 CRC cells engineered to overexpress NANOG (HCT116^{GFP/Nanog}) with control cells (HCT116^{GFP}) [10]. Untreated HCT116^{GFP/Nanog} cells generated on average ~30 % more spheres than the HCT116^{GFP} controls (Supplemental Fig. S5A–C) and were more sensitive to curcumin exposure (Fig. 5A–B). There have been reports that curcumin promotes autophagic survival of a subset of CSCs and resistance to treatment, following long-term *in-vitro* exposure for ~30 days [20]. To ascertain whether prolonged curcumin treatment of HCT116^{GFP/Nanog} cells led to resistance, cells were treated on alternate days for 2 weeks (top row, Fig. 5A). There were at least ~60 % fewer spheres present in wells treated with >1 μM curcumin containing the HCT116^{GFP/Nanog} cells, with ~35 % fewer spheres in HCT116^{GFP} controls relative to each solvent control, though the untreated HCT116^{GFP/Nanog} cells generated more spheres at the outset (Fig. 5B). Subsequently, spheres were harvested and re-plated to monitor sphere formation in the absence of curcumin. The growth of cells was considerably delayed in a concentration-dependent manner compared to the solvent control (Supplemental Fig. S5D). At day 83, spheroids were

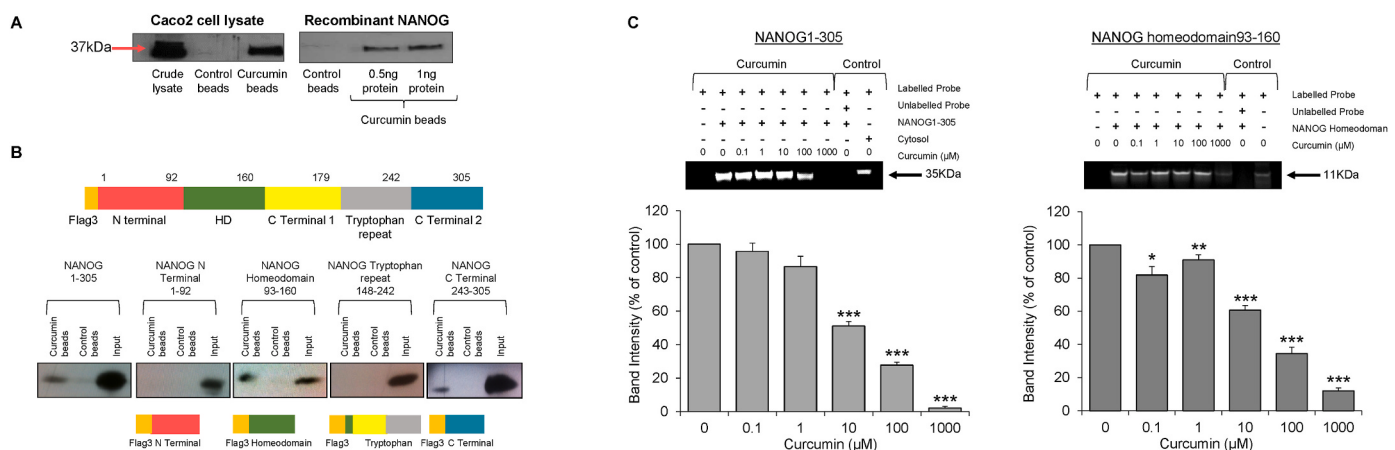


Fig. 4. Curcumin directly binds to NANOG protein and inhibits NANOG-DNA interactions. (A–B) Affinity pull-down assays were performed using curcumin-coupled beads incubated with Caco2 whole cell lysate, commercially available recombinant NANOG protein and flag-tagged truncated proteins synthesized in house. (C) EMSAs were conducted using recombinant full length NANOG protein and the homeodomain incubated with curcumin (0–1000 μM) and DNA probes modified with 5'-infra-red 700 labels containing the homeodomain binding motif TAATGG. Unlabelled forward probe was used in a competition assay and cytosol from Caco2 cells was the positive control. Bands were quantified and the bound fraction compared with 0 μM curcumin. Three independent experiments were performed. Values represent mean + SEM. Significant differences relative to control are shown, where * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

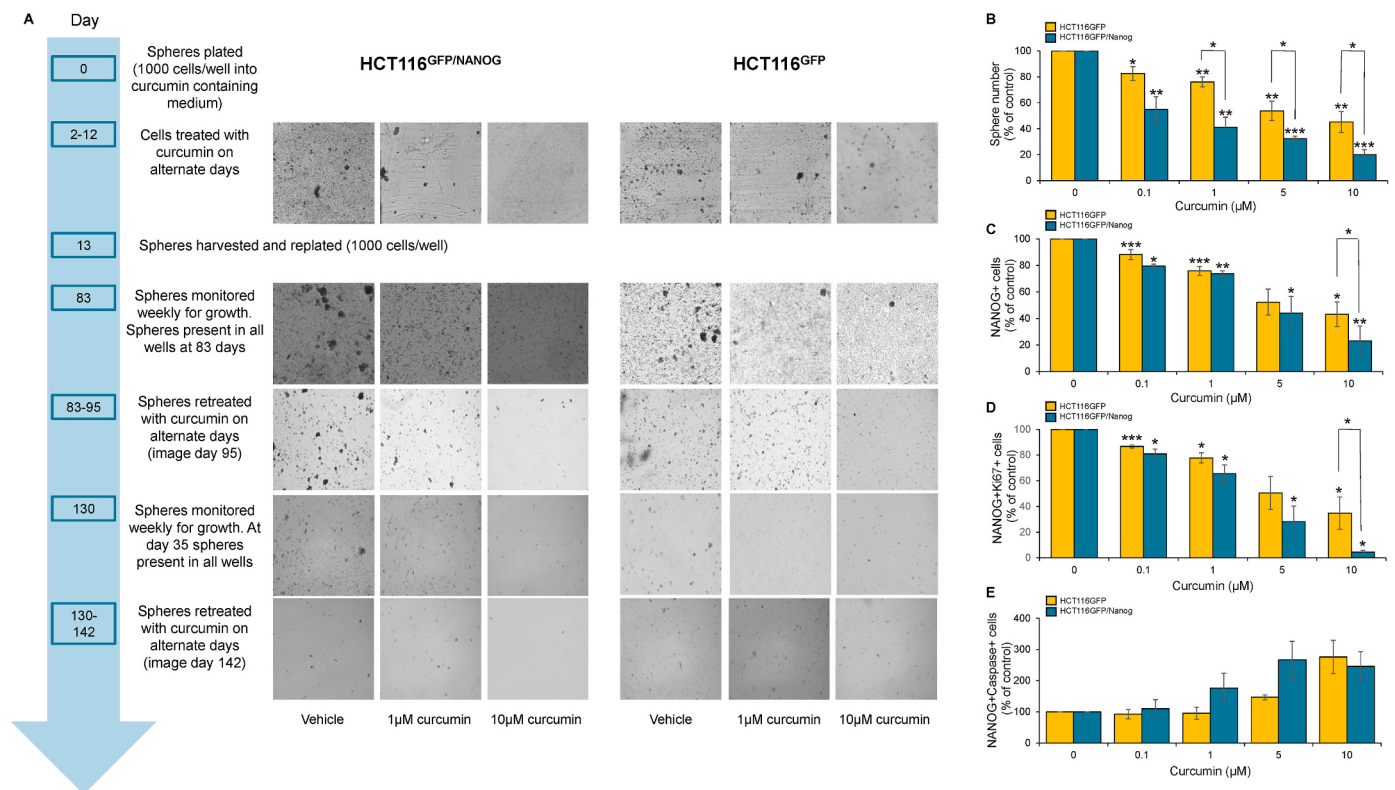


Fig. 5. Curcumin preferentially targets NANOG overexpressing HCT116^{GFP/Nanog} cells. (A–B) Single HCT116^{GFP/Nanog} and HCT116^{GFP} cells were plated in sphere medium and treated with curcumin or vehicle (0–10 μM) on alternate days for two weeks. At day 13, one plate of representative spheres for each concentration was harvested and counted blind. The remaining plates were harvested and cells replated, then at day 83, cells underwent a second two-week treatment with curcumin *in-situ*. Spheres reformed in all wells by ~35 days following treatment, demonstrating the cells had not become resistant, and subsequently underwent a third round of treatment (days 130–142). Representative images are shown from triplicate experiments (A, top row) and single replicates (A, bottom four rows), x5 magnification. (C–E). Cells were plated and treated with curcumin for two weeks, harvested and analysed by FACS to quantify the NANOG⁺ (C), NANOG⁺/Ki67⁺ (D) and NANOG⁺/Caspase⁺ (E) populations. (B–E) At least 3 independent experiments were performed. Values represent mean ± SEM, with significant differences relative to control shown by *p < 0.05, **p < 0.01, ***p < 0.001.

visible in all wells and were exposed to a second curcumin treatment *in-situ* for 2 weeks. HCT116^{GFP/Nanog} spheroids remained sensitive to curcumin and were reduced in number compared to solvent control; similar activity was apparent in the HCT116^{GFP} spheres, but more spheres remained, which is consistent with the over-expressing cells having greater sensitivity. Following a further ~35 days, spheres were visible in all wells and were treated again with curcumin *in-situ* for a third period, with minimal spheres present at the end of this exposure. This illustrates that the spheres did not acquire resistance (Fig. 5A Days 83–142) after multiple treatments, over a prolonged period of time and curcumin maintained its efficacy.

FACS analysis of spheres treated with curcumin for 2 weeks showed that the proportion of NANOG-expressing cells significantly decreased in both cell lines in a concentration dependent manner (Fig. 5C). Curcumin appeared to specifically target the proliferating fraction of CSCs, defined by co-expression of NANOG⁺/Ki67⁺, with a greater inhibitory effect in the HCT116^{GFP/Nanog} overexpressing cell line compared to the HCT116^{GFP} control cells. Furthermore, there was a trend towards an increase in the non-stem proliferating fraction (NANOG[−]/Ki67⁺ population) (Fig. 5D, Supplemental Fig. S5E) and increasing apoptosis in these cells, detected by Caspase⁺/NANOG⁺ co-expression. However, the magnitude of this effect was not sufficient to fully account for the loss of NANOG⁺ cells (Fig. 5E).

2.5. Curcumin targets NANOG⁺ Ki67⁺ CSCs in CRC and adenoma patient-derived explants

To examine whether the effects of curcumin on stem-cells translated

to intact human tissues, patient-derived explants (PDEs) from 20 individuals were cultured in curcumin-containing media for 24 h (Supplemental Table S3A). Following this short-term exposure, the majority of samples (16/20) demonstrated a response to curcumin although the nature of the dose-response relationships varied between patients, consistent with the observations in sphere cultures; 7 displayed a broadly linear-dose response showing a reduction in NANOG⁺ cells with an increasing concentration of curcumin and 9 showed a non-linear/U-shaped response, with greater effects at lower concentrations. Adenoma tissues accounted for 3 out of the 16 patients and these exhibited either a linear (n = 1) or non-linear (n = 2) response to curcumin (Supplemental Fig. S6A–D). The remaining 4 patients showed either no detectable response or an increase in the proportion of NANOG⁺ cells (non-responders). When evaluating the maximal response, either a reduction or increase in the NANOG⁺ fraction after curcumin incubation, half of all the 20 patients had a reduction of greater than 35 %, with 2 patients having just ~10 % of their NANOG fraction remaining relative to the respective solvent control (Fig. 6A). When the proliferating component of NANOG-expressing cells was considered, only 2/20 samples were non-responders and in 5 patients the NANOG⁺Ki67⁺ population was eradicated (Fig. 6B). When evaluating all PDEs together as an average response, curcumin significantly reduced the NANOG⁺Ki67⁺ population following treatment with 0.1 and 1 μM curcumin (Fig. 6C). The lack of significant effects at the two highest concentrations when results are combined is probably due to the mixed dose-response relationships observed across the different PDEs and reflects the non-linear/U-shaped activity exhibited by curcumin in almost half of the patient samples (Supplemental Fig. S6B–D). Overall,

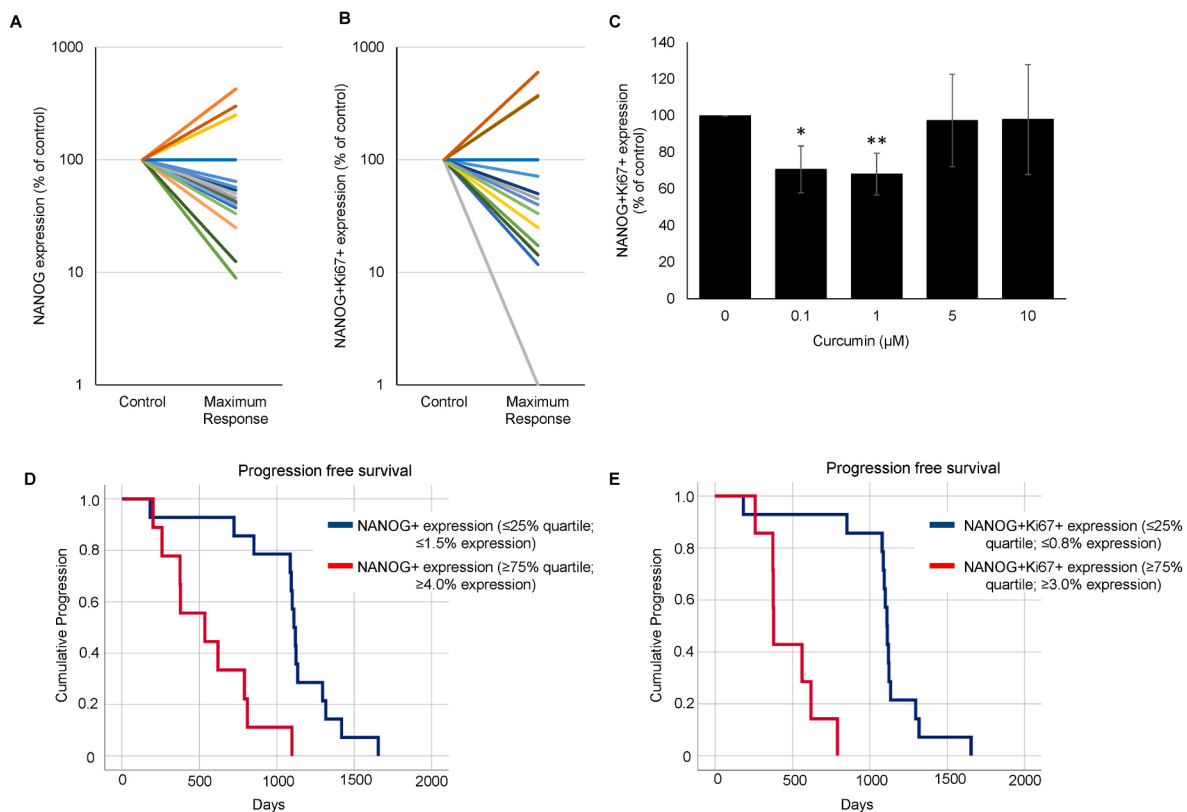


Fig. 6. Curcumin targets NANOG⁺ Ki67⁺ in CRC and adenoma patient-derived explants (PDEs). **(A–B)** PDEs (n = 20) were cultured in explant media overnight and treated with curcumin or solvent only for 24 h (0–10 μM). Tissues were harvested and single cells created and stained. The maximal change in NANOG⁺ **(A)** or NANOG⁺ Ki67⁺ **(B)** expression relative to the respective control is shown for each patient sample; full results over the entire concentration range for each individual are presented in [Supplementary Fig. 7](#). **(C)** The mean (±SEM) response across the 20 PDEs for NANOG⁺ Ki67⁺ expression demonstrates significant reductions in this population with 0.1 and 1 μM curcumin (*p < 0.05, **p < 0.01). **(D–E)** CRC tissues were profiled for NANOG⁺ and NANOG⁺ Ki67⁺ expression (n = 46). The top and bottom quartile were compared for progression free survival. **(D)** In the NANOG⁺ population ≤25 % quartile, the median survival was 1111 days whilst in the ≥75 % quartile it was 536 days (p < 0.001). **(E)** In the NANOG⁺ Ki67⁺ population ≤25 % quartile, the median survival was 1111 days compared to the ≥75 % quartile where it was 379 days (p < 0.001).

these results suggest that curcumin targets the stem-like component within PDEs as it had no significant effect on the percentage of NANOG⁺ cells relative to matched control ([Supplemental Fig. S6E](#)).

CRC has been characterised based on transcriptomic profiling into four consensus molecular subtypes (CMS), which are clinically significant in terms of patient survival; immune, canonical, metabolic and mesenchymal. We categorised the PDE samples using established immunohistochemistry methods and found curcumin to have broad spectrum activity across all subtypes ([Supplemental Table S3B](#)) [26].

To explore the potential clinical significance of a reduction in the proportion of NANOG⁺ expressing cells and the proliferative compartment within this population, tissues from 46 cancer patients were profiled by FACS analysis and these parameters were correlated with progression free survival (PFS) ([Supplemental Table S1B](#)). The top 25 % NANOG⁺ and NANOG⁺ Ki67⁺ expressers were compared with the bottom 25 % ([Fig. 6D and E](#)). With respect to the NANOG⁺ population, patients in the bottom quartile had a median survival of 1111 days, whilst for those in the top quartile it was just 536 days (p < 0.001) ([Fig. 6D](#)). The difference in median PFS for patients in the top and bottom quartiles for NANOG⁺ Ki67⁺ expression was even greater, at 1111 versus 379 days, respectively (p < 0.001). These findings suggest that reducing the proportion of stem-like cells characterised by NANOG-expression, and particularly those that are actively proliferating may lead to significant clinical benefit.

2.6. Curcumin promotes differentiation of human colorectal CSCs

The loss of NANOG-expressing CSCs in response to curcumin was not fully explained by the small amount of apoptosis-induced in HCT116 cell lines. Instead, the trend towards an increase in proliferating non-stem cells in these cultures, albeit not significant apart from at the 1 μM concentration in HCT116^{GFP} control cells ([Fig. S5E](#)), could be indicative of enhanced differentiation. Therefore, to investigate whether short-term exposure to curcumin may push NANOG⁺ cells towards lineage differentiation, gene-expression changes in HCT116^{GFP/Nanog} cells were evaluated. Principal component analysis revealed distinct control and curcumin treated populations at 72 h but not at 24 h, suggesting it takes several days for the effects of curcumin to fully manifest ([Fig. 7A and Supplemental Fig. S7](#)). The shorter timepoint of 24 h was selected to align with the PDE culture experiments, but it was not possible to conduct longer term incubations of curcumin with PDEs to match the 72 h timepoint, due to reduced tissue viability. Fifty-eight differentially expressed genes (FDR p < 0.05) were identified between control and curcumin treated cells at 24 h, and 760 genes at 72 h ([Fig. 7B](#)). Gene Set Enrichment Analysis (GSEA) revealed significant upregulation (p < 0.05) of pathways involved in EMT and intestinal differentiation, whilst stem-cell related genes were down regulated (p < 0.05) ([Fig. 7C](#)). GSEA analysis using stem-cell and differentiation signatures from published literature [27–29] demonstrated that stem-cell signature genes were down regulated by curcumin; this occurred to a greater extent at 24 h compared to 72 h, and was accompanied by upregulation of differentiation signature genes, with a stronger effect at the 72 h time point. A loss

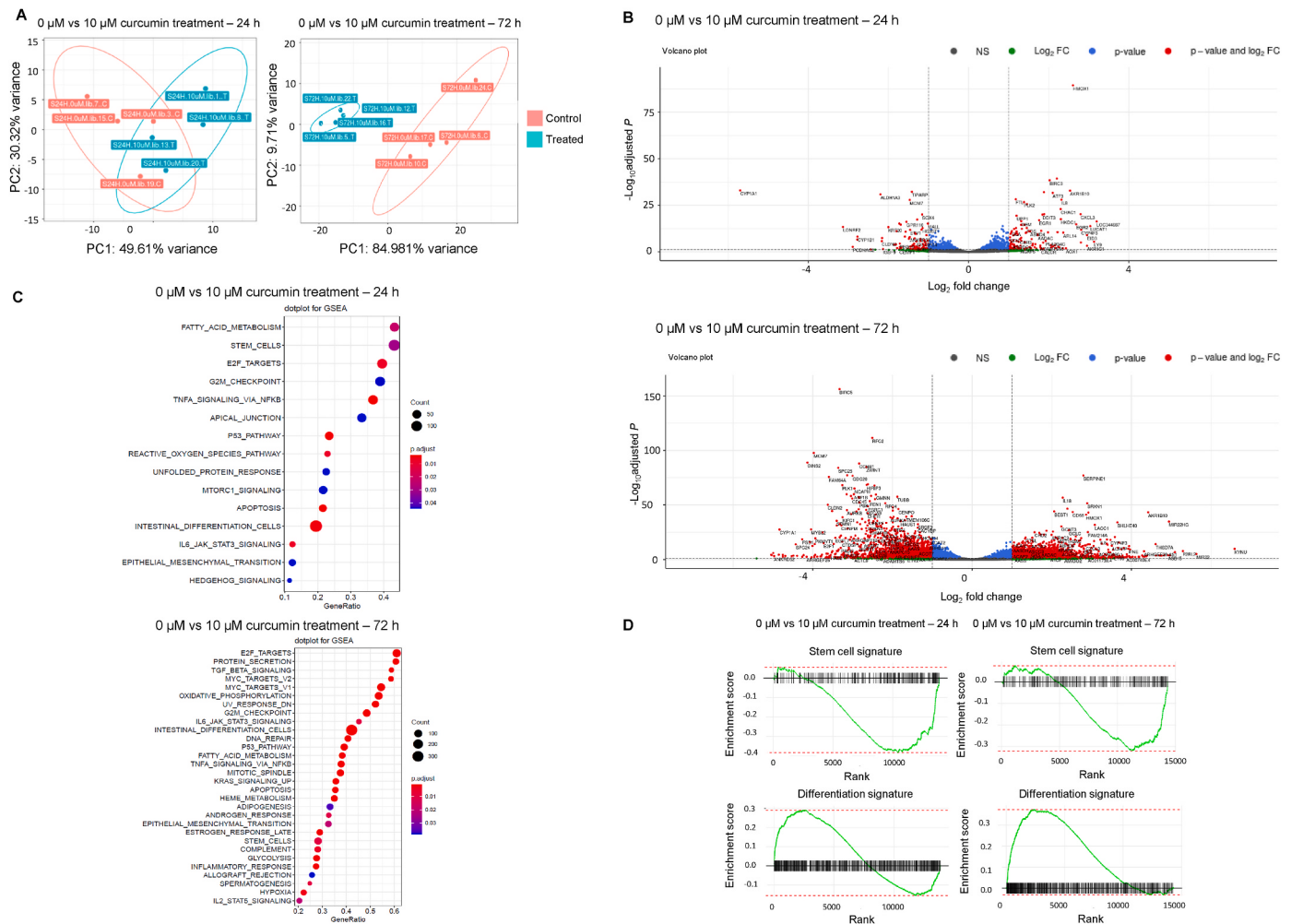


Fig. 7. Curcumin promotes plasticity of human colorectal cancer stem-like cells into differentiated cells. **(A)** HCT116^{GFP/Nanog} spheroids were treated with 10 μ M curcumin for 24 and 72 h prior to harvesting, extracting RNA and conducting RNAseq analysis. Principal component analysis of the datasets demonstrated an overlap between control and curcumin treated samples at 24 h, with distinct clustered populations at 72 h. **(B)** Unsupervised analysis identified 58 and 760 genes which were differentially expressed (FDR $p < 0.05$) between solvent control and curcumin treated HCT116^{GFP/Nanog} cells at 24 and 72 h, respectively. **(C)** Hallmark pathways were reviewed using normalised enrichment scores from GSEA functions. **(D)** Targeted review of known stem-cell and differentiation signatures demonstrated greater down regulation of stem-cell signature genes at 24 compared to 72 h, whilst there was greater upregulation of differentiation signature genes at the longer time point.

of stem-cell signature suggests reduced *NANOG* activity. Taken together these findings indicate that curcumin inhibits the propagation and growth of stem-cells within premalignant and cancer tissue by promoting an environment supporting CSC lineage differentiation (Fig. 7D).

3. Discussion

Curcumin is a pleiotropic low-cost compound with a favourable safety profile, targeting a diverse range of anti-cancer and non-cancer related pathways [12]. Its promiscuity offers the possibility of simultaneously targeting multiple dysregulated processes in a developing tumour, prior to introduction of greater complexity and heterogeneity that may ultimately lead to resistance. Additionally, broad activity may be desirable in a prevention setting as the genetic drivers within premalignant cells will be unknown for the majority of sporadic cancers, so interventions need to be capable of intercepting multiple subtypes of tumours.

In deciphering key mechanisms of action of curcumin in human tissues, it was important to employ clinically-relevant concentrations. Curcumin is renowned for its low systemic bioavailability due to poor absorption and rapid metabolism; however, relatively high concentrations of the intact parent compound exist in the gastrointestinal tract

after oral ingestion. We have previously demonstrated that daily administration of 2.35 g to patients furnishes colorectal tissue concentrations of $\sim 50 \mu$ M, with circulating plasma levels either undetectable or in the region of $\sim 0.05 \mu$ M [11]. These exposures align with the steady state levels previously reported in the intestine of mice administered 0.2 % in their diet ($\sim 100 \mu$ M), which is an efficacious dose in *Apc*^{Min/+} mice [17]. Although we did not measure systemic or intra-tumoral concentrations of curcumin in the patient derived xenografts, our previous pharmacokinetic study using the same phytosomal formulation revealed that it generates ~ 4 x higher plasma concentrations in mice than standard curcumin [16]. Therefore, given that only trace levels of around 5 nmol/L are detectable in the plasma of mice administered 0.2 % standard curcumin [17], it might be estimated that systemic concentrations in the xenograft mice were in the region of $\sim 0.02 \mu$ M; encouragingly, this is lower than concentrations found to inhibit stem cell proliferation in the three different human models used.

Ultimately, if curcumin is to be used for cancer prevention it is likely to necessitate long-term administration, which could present compliance problems at doses above ~ 2 g due to the number and size of capsules required; it would therefore be advantageous if lower doses had activity. Consequently, it was important to identify the lowest effective curcumin concentrations to ensure meaningful translation to a clinical

setting. This is paramount for preventive therapies, as there are examples of supraphysiological concentrations being tested, making interpretation of data difficult and translational relevance questionable [30]. As indicated above, our data demonstrate that low, clinically achievable concentrations of curcumin have biological activity in human tissues that could lead to health benefits. Moreover, we generally observed a non-linear dose response, where lower exposures elicit a greater response than higher, highlighting the need for greater understanding when selecting doses for clinical evaluation - a phenomenon previously demonstrated for other dietary agents in this context [31].

Another consideration was use of platforms that are able to predict response to therapy in a clinical setting. As a result, human tissues cultured *ex-vivo* as 3D spheroids or PDEs were employed, as they have demonstrated clinical predictivity, and adenoma tissue was utilised where possible [32,33]. Premalignant or early-stage CRC tissues are likely to be more representative of the *in-vivo* situation than tissues derived from advanced disease and we also conducted long-term incubations with low concentrations in our initial experiments to mimic a clinical paradigm where low doses would be administered chronically.

Various protein biomarkers have been proposed to identify subpopulations of colorectal CSCs that are functionally and phenotypically heterogeneous and may arise from crypt stem cells or alternatively, from compartments outside of the crypt base columnar cells, following dedifferentiation. In this study curcumin was found to reduce different populations of cancer/premalignant stem cells defined by four biomarkers, ALDH activity as well as CD133, DCAMKL-1 and NANOG expression. Whilst ALDH^{high} activity characterises cells that encompass subpopulations also expressing CD133 and NANOG individually (Figs. 1A and 3D), less is known about the overlap with DCAMKL-1 expression, which was not investigated in this study. It has however, previously been shown that treatment of HCT-116 cell-derived xenografts with DCAMKL-1-siRNA attenuates the expression of NANOG and ALDH1A1 as well as the target protein; moreover, in the same model, curcumin also significantly reduced the expression of these three stem cell markers in tumour tissue [20].

Exploiting CSC plasticity as a therapeutic target to reduce the population of cells with stem-like characteristics, is an attractive proposition in premalignant adenoma tissues, where high levels of ALDH^{high} cells and NANOG-expression were detected. Since NANOG is found in embryonic and cancerous cells but not in normal healthy tissues, it presents a highly specific target in adults [21]. Our data indicate that curcumin reduces the proliferating fraction of NANOG⁺ cells and induces an environment that favours CSC lineage differentiation over self-renewal, with NANOG potentially being a key effector.

Daily curcumin delivered in the diet also reduced the number of DCAMKL-1⁺ labelled stem cells in the intestines of *Apc*^{Min/+} but not wild type mice, which aligns with its inhibitory effects against polyp formation in the same animals [17] and is also consistent with the anti-tumour activity observed in a HCT116 xenograft model, coupled with reduced tissue expression of DCAMKL-1 [20]. In this latter study, treatment of mice with DCAMKL-1-siRNA had a stronger inhibitory effect on the growth of established tumours than curcumin alone administered by injection every 2 days, whilst the combination provided additional benefit, leading the authors to suggest that curcumin-induced autophagy allows a small subset of DCAMKL-1⁺ CSCs to continue proliferating and maintain tumour mass [20]. Although autophagy was not examined as an end point in the present study, our long-term spheroid experiment (Fig. 5A) revealed no evidence of resistance to curcumin developing over the 142 days in culture, at the low concentrations used.

Curcumin has received criticism as a pan-assay interference compound (PAIN), yielding misleading results in traditional molecular drug screens due to interference with assay readouts, rather than through specific compound/target interactions [34]. Whilst this possibility needs acknowledging, it should not preclude the pursuit of curcumin or allow disregard of promising preclinical or clinical results; rather, experiments

must be well-designed and conducted with rigour to minimise false positives [35]. We employed several independent methods to demonstrate a direct interaction between curcumin and NANOG, initially using affinity pull down assays with curcumin-coupled beads to show binding to endogenous NANOG in cell lysates, recombinant full-length protein and fragments containing the homeodomain and C-terminus. Binding was further confirmed using EMSA and we discovered that curcumin interferes with the ability of NANOG to bind DNA. Although curcumin treatment reduced total NANOG levels in cell lysates, we found no evidence it affects phosphorylation status or protein stability using a cycloheximide assay (data not shown), as has been reported for aspirin [36]. Instead, these results are consistent with a reduction in the proportion of NANOG-expressing cells, as was subsequently observed in the PDE samples treated with curcumin.

Targeting transcription factors such as NANOG has been challenging due to their lack of enzymatic activity or well-defined pockets, but is improving through a growing set of inhibitors of this class of proteins, to which our work contributes [37]. For many single target cancer therapies, development of resistance and treatment-limiting toxicity is often prevalent. There is significant interest from the pharmaceutical industry in developing drugs against CSCs [9], but their natural plasticity could mean that a low toxicity therapy that targets both CSCs and non-CSCs may provide greater clinical benefit. This, coupled with the broad range of activity curcumin demonstrated across all four CRC subtypes supports its evaluation in a prevention setting on a non-selected basis. However, it is incumbent to recognise that a recent double-blind placebo-controlled trial of curcumin (3 g/day) in individuals with familial adenomatous polyposis (FAP) failed to achieve its primary objective of suppression and/or growth of new polyps over a 12-month period [38]. In contrast, a previous study showed polyp regression when a lower dose of curcumin was combined with piperine [38]. There are a number of factors that may account for the lack efficacy in this specific population. Firstly, although classical FAP is caused by germline mutations in the APC gene, which is altered in ~80 % of sporadic CRCs, the disease presents differently. Individuals typically develop hundreds to thousands of colorectal polyps from their teenage years, compared with the decades it takes for sporadic CRC to form. Consequently, trials in FAP patients are often seeking a preventive effect with regression of established disease to reduce overall polyp burden over a short period, which can render detection of potentially efficacious interventions challenging. It is worth noting that trials of aspirin in individuals with FAP have also reported a lack of clinical benefit [39], despite the well-established ability of aspirin use to reduce the risk of CRC in the general population and individuals with Lynch syndrome [7,40]. Importantly, the protective effects of aspirin take ~5–10 years to manifest after initiation of therapy; this delay may partly explain why aspirin failed to significantly reduce polyp number in short-term studies involving patients with FAP and highlights that negative outcomes in such trials should not discount further clinical evaluation in a different population. It remains to be tested whether the ability of curcumin to direct proliferating CSCs within premalignant adenomas or early cancers towards differentiation translates to clinical efficacy in high-risk populations. In this respect there is future potential for curcumin to feed into a phase 2 signal-seeking study within the COLO-PREVENT trial platform (ISRCTN13526628) to assess whether it can prevent sporadic colorectal polyp development and further interrogate the mechanisms of action in patient tissues, including through the analysis of NANOG expressing proliferating cells and markers of differentiation. To enable future clinically-informed dosing recommendations, such trials should ideally include a range of doses.

4. Materials and methods

4.1. Clinical samples

Human colorectal cancer and adenoma tissues were obtained as part

of an ethically approved study (UNOLE 0472/REC reference 14/WA/1166, received October 23, 2014). All procedures were performed in compliance with relevant laws and institutional guidelines. The privacy rights of all participants have been observed and informed consent was obtained for the work conducted. Tissue from 66 patients was used to profile ALDH activity and CD133⁺ expression. A further 50 patient samples were used to assess NANOG⁺ and NANOG⁺Ki67⁺ expression, and 20 tissue samples were used for explants.

4.2. Cell culture, in vitro methods and biophysical assays

Full details on *ex vivo* spheroid and 2D-cell culture and incubation with curcumin are provided in Supplementary materials, along with descriptions of the protein pull-down methodology and biophysical assays.

4.3. Flow cytometry

To assess ALDH activity and CD133⁺ expression, Aldefluor assay kits (Stem Cell Technologies, UK) and APC-conjugated mouse anti-human CD133 antibodies were used, with PE-conjugated mouse anti-human ESA antibody (Miltenyi Biotech, UK). For analysis of NANOG⁺ and NANOG⁺Ki67⁺ expression, cells were fixed and permeabilised using Cell Cignalling buffer set A (Miltenyi Biotech, UK). PE-conjugated mouse anti-human Ki67 antibody, APC-conjugated mouse anti-human NANOG antibody and anti-human IgG1 (Miltenyi Biotech, UK) was used as a negative control (see [Supplementary Fig. S8](#) for gating strategy). Analysis was performed on a BD FACS Aria II and FACSDiva v6.1.3 was used for data acquisition and analysis, with 10,000 events recorded for each sample.

4.4. Patient derived explants

PDEs were treated with curcumin (Sigma, UK) (0–10 μ M) or vehicle alone for 24 h [41]. PDEs were assessed for NANOG⁺ and NANOG⁺Ki67⁺ expression using FACS analysis. CRC tissues were classified into CMS using an automated classifier [26].

4.5. In vivo studies

All *in vivo* studies met UKCCCR standards and were performed under project license 60/4370, with approval from the University of Leicester Ethics Committee and in accordance with their guidelines. Isolated ALDH^{high} cells were transplanted subcutaneously into male non-obese diabetic/severe combined immunodeficiency (NOD/SCID, Harlem Laboratories) mice. The cells originated from a single patient with CRC, and tissue was first expanded by serial transplantation in multiple NOD/SCID mice. Cells were harvested, pooled and FACS sorted. Mice were maintained on irradiated powdered diet containing either 0.9 % Epikuron (n = 19), or 1.13 % Meriva, equivalent to 0.2 % curcumin (n = 20). A single-cell suspension of the ALDH^{high} CSCs was mixed with Matrigel 1:1 (v/v) and injected subcutaneously (100 μ L) into the right and left mid-abdominal areas. Body weight and tumour size were measured twice weekly for 16–20 weeks. After tumours reached ~5 % of the animal's body weight (~17 mm diameter) or earlier if the tumour ulcerated, animals were sacrificed. Researchers were blinded to treatment.

For analysis of DCAMKL-1 expression, sections were obtained from archived *Apc*^{Min/+} mouse intestine samples from our previous study [17]. Endogenous peroxidase activity was blocked using Avidin/Biotin blocking kit (Dako, UK). Sections were exposed to primary rabbit polyclonal anti-DCAMKL-1 (Perkins, UK) or negative control (blocking peptide targeted against DCAMKL-1 epitope) (Abcam, UK).

4.6. Statistical analysis

Data were visualised using GraphPad Prism 9, Microsoft Office 2016, SPSS v25.0, FACSDiva v6.1.3, chemiluminescent western blot imaging system (Syngene) and NDP view.2. RNASeq data was analysed using hg19 GENCODE. For statistical analysis, Student t-test, Mann-Whitney *U* Test or Breslow (Generalized Wilcoxon) was performed and *p* < 0.05 considered significant. For correlation analysis Pearson was performed, with *p* < 0.05 being significant. At least 3 independent experiments were performed for biological and biophysical experiments.

CRedit authorship contribution statement

Sam Khan: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Ankur Karmokar:** Conceptualization, Data curation, Formal analysis, Investigation, Resources, Writing – original draft. **Lynne Howells:** Conceptualization, Supervision, Writing – review & editing. **Robert G. Britton:** Data curation, Investigation, Methodology, Resources, Writing – review & editing. **Emma Parrott:** Methodology, Writing – review & editing. **Raquel Palacios-Gallego:** Data curation, Formal analysis, Writing – review & editing. **Cristina Tufarelli:** Conceptualization, Supervision, Writing – review & editing. **Hong Cai:** Formal analysis, Writing – review & editing. **Jennifer Higgins:** Data curation, Writing – review & editing. **Nicolas Sylvius:** Investigation, Resources, Writing – review & editing. **Kevin West:** Formal analysis, Investigation, Writing – review & editing. **Angus McGregor:** Resources, Writing – review & editing. **David Moore:** Formal analysis, Writing – review & editing. **Selena G. Burgess:** Methodology, Writing – review & editing. **Mark W. Richards:** Methodology, Writing – review & editing. **Anja Winter:** Methodology, Resources, Writing – review & editing. **Zahirah Sidat:** Methodology, Writing – review & editing. **Nalini Foreman:** Methodology, Project administration, Writing – review & editing. **Sanne T. Hoorn:** Investigation, Methodology, Software, Writing – review & editing. **Louis Vermeulen:** Investigation, Methodology, Software, Writing – review & editing. **Baljit Singh:** Resources, Writing – review & editing. **David Hemingway:** Resources, Writing – review & editing. **Michael Norwood:** Resources, Writing – review & editing. **Andrew Miller:** Resources, Writing – review & editing. **Kirsten Boyle:** Resources, Writing – review & editing. **Carmen Ho:** Resources, Writing – review & editing. **Muhammad I. Aslam:** Resources, Writing – review & editing. **Richard Bayliss:** Methodology, Resources, Supervision, Writing – review & editing. **Abdolrahman S. Nateri:** Methodology, Resources, Writing – review & editing. **Alessandro Rufini:** Investigation, Visualization, Writing – review & editing. **Andreas Gescher:** Funding acquisition, Writing – review & editing. **Anne L. Thomas:** Funding acquisition, Supervision, Writing – review & editing. **William P. Steward:** Funding acquisition, Supervision, Writing – review & editing. **Karen Brown:** Conceptualization, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Data and materials availability

RNASeq data will be deposited in the European Nucleotide Archive following publication. Where remaining archived tissue is available from the murine models, this could be shared, subject to appropriate agreements.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: DM reports speaker fees from AstraZeneca, Eli Lilly and Takeda, consultancy fees from AstraZeneca, Thermo Fisher, Takeda, Amgen, Janssen, MIM Software, Bristol-Myers Squibb and Eli Lilly and has received

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Authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2025.217885>.

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