RESEARCH ARTICLE

Vitamin D treatment induces in vitro and ex vivo transcriptomic changes indicating anti-tumor effects

Peter G. Vaughan-Shaw1,2 | James P. Blackmur1,2 | Graeme Grimes1,2 | Li-Yin Ooi2,3 | Anna M. Ochocka-Fox1,2 | Karen Dunbar1,2 | Alex von Kriegsheim2 | Vidya Rajasekaran1,2 | Maria Timofeeva2,4 | Marion Walker1,2 | Victoria Svinti2 | Farhat V. N. Din2 | Susan M. Farrington1,2 | Malcolm G. Dunlop1,2

1MRC Human Genetics Unit, Institute of Genetics and Cancer, University of Edinburgh, Edinburgh, UK
2Cancer Research UK Edinburgh Centre, Institute of Genetics and Cancer, University of Edinburgh, Edinburgh, UK
3Department of Pathology, National University Hospital, National University Health System, Singapore City, Singapore
4DIAS, Danish Institute for Advanced Study, Department of Public Health, University of Southern Denmark, Odense, Denmark

Correspondence
Malcolm G. Dunlop and Susan M. Farrington, Cancer Research UK Edinburgh Centre, Institute of Genetics and Cancer, University of Edinburgh, Edinburgh EH4 2XU, UK. Emails: malcolm.dunlop@ed.ac.uk and susan.farrington@ed.ac.uk

Funding information
This work was supported by funding for the infrastructure and staffing of the Edinburgh CRUK Cancer Research Centre; CRUK programme grant C348/A18927 (MGD/SMF). PVS was supported by MRC Clinical Research Training Fellowship (MR/M004007/1), a Research Fellowship from the Harold Bridges bequest and by the Melville Trust for the Care and Cure of Cancer. The work received support from COST Action BM1206. LYO is supported by a Cancer Research UK Research Training Fellowship (C10195/A12996). JB is supported by an Edinburgh Clinical Academic Track (ECAT) linked Cancer Research UK Clinical

Abstract
Vitamin D deficiency is associated with risk of several common cancers, including colorectal cancer (CRC). Here we have utilized patient derived epithelial organoids (ex vivo) and CRC cell lines (in vitro) to show that calcitriol (1,25OHD) increased the expression of the CRC tumor suppressor gene, CDH1, at both the transcript and protein level. Whole genome expression analysis demonstrated significant differential expression of a further six genes after 1,25OHD treatment, including genes with established links to carcinogenesis GADD45, EFTUD1 and KIAA1199. Furthermore, gene ontologies relevant to carcinogenesis were enriched by 1,25OHD treatment (e.g., ‘regulation of Wnt signaling pathway’, ‘regulation of cell death’), with common enriched processes across in vitro and ex vivo cultures including ‘negative regulation of cell proliferation’, ‘regulation of cell migration’ and ‘regulation of cell differentiation’. Our results identify genes and pathways that are modifiable by calcitriol that have links to CRC tumorigenesis. Hence the findings provide potential mechanism to the epidemiological and clinical trial data indicating a causal association between vitamin D and CRC. We suggest there is strong rationale for further well-designed trials of vitamin D supplementation as a novel CRC chemopreventive and chemotherapeutic agent.

Abbreviations: 1,25-OHD, 1,25-dihydroxyvitamin D; CRC, colorectal cancer; DNA, deoxyribonucleic acid; FAP, familial adenomatous polyposis; FC, fold-change; GO, gene ontology; GSEA, gene set enrichment analysis; qRT-PCR, quantitative reverse transcription–PCR; RCT, randomized control trial; RNA, ribonucleic acid; SNP, single nucleotide polymorphism; VDR, vitamin D receptor gene.

Susan M. Farrington and Malcolm G. Dunlop are joint authors at these positions.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2021 The Authors. The FASEB Journal published by Wiley Periodicals LLC on behalf of Federation of American Societies for Experimental Biology.
1 | INTRODUCTION

Vitamin D deficiency is associated with risk of several common cancers, with the strongest available evidence supporting a link between vitamin D and colorectal cancer.1,2 Observational data implicating vitamin D deficiency in CRC etiology or survival are limited by potential bias as environmental risk factors associated with CRC are also associated with vitamin D status (i.e., co-causality; e.g., physical activity), while the colorectal cancer or its treatment may impair vitamin D status (i.e., reverse causation). Indeed, a definitive causal relationship remains unproven,3 with existing mendelian randomization (MR) studies hampered by weak genetic instrumental variables4–5 and large trials (e.g., VITAL Trial6 and ViDA study7) failing to demonstrate an effect of vitamin D supplementation on CRC risk. However, a recent randomized-control trial (RCT) reported an association between supplementation, vitamin D receptor genotype and risk of colorectal adenoma, supporting the premise that the beneficial effect may be causal.8 Meanwhile, vitamin D-related genetic variation has been shown to influence the association between 25-OHD level and CRC survival,9–11 and a recent meta-analysis of RCT data strongly supports a causal effect for vitamin D supplementation on CRC mortality.12,13

A variety of pre-clinical experimental studies have provided some understanding of the biological functions of vitamin D in relation to cancer initiation or progression. In vitro studies using CRC cell lines have demonstrated the influence of vitamin D on relevant cellular processes including proliferation and apoptosis.14 Meanwhile, differential gene expression is reported in CRC and adenoma tissue compared to normal colorectal tissue,15,16 with genes involved in metabolism, transcription and translation and cellular processes commonly altered17 and vitamin D is known to influence gene expression through activation of the ligand-activated transcription factor VDR.18–20 Palmer et al., report induction of CDH1 in response to 1-alpha,25(OH)2D(3) treatment of SW480 (CRC) cell lines,21 providing possible mechanism via influences on E-cadherin and Wnt/β-catenin signaling pathway. Yu et al., report impacts of calcitriol on cell migration, apoptosis, autophagy, and epithelial-mesenchymal transition, sensitizing CRC cells to ionizing radiation.22 Other reports demonstrate similarly broad influences on gene expression with vitamin D impacting cancer cell growth in vitro.23,24

The limitations of comparative cell line studies in the investigation of gene expression are widely acknowledged; namely that accumulated cellular genomic and karyotypic abnormalities may significantly influence results from experimental studies. It is not surprising, therefore that epithelial organoids are now emerging as a non-aberrant ex vivo model system for the investigation of tumor initiation. The colonic epithelial organoid culture is a long-term culture system derived from intestinal crypt stem cells that aims to maintain basic crypt physiology and display the hallmarks of intestinal epithelium in terms of architecture, cell type composition, and self-renewal dynamics. A small number of studies have now demonstrated gene expression changes in patient-derived organoids treated with vitamin D, supporting the use of this as a model for vitamin D intervention studies.25–27

Here, we investigate the effects of vitamin D on in vitro and ex vivo epithelial cell gene expression and assess for links to anti-tumor effects as a putative mechanism of action of vitamin D, as a chemopreventive or chemotherapeutic agent in CRC.

2 | METHODS

2.1 | Cell culture and treatment

Established colorectal cancer cell lines were used (SW480, LS174T, SW48, DLD1, HCT116, COLO205, T84, LOVO, VACO425, CaCO-2), cultured under standard conditions.28 In brief, Dulbecco’s Modified Eagle Medium was used (high glucose, with L-glutamine; DMEM) (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with Fetal Calf Serum (10%), or charcoal-treated FCS (10%, for treatments; Sigma, St Louis, MO, USA) and Penicillin/Streptomycin (100 IU/ml penicillin and 100 µg/ml streptomycin 1%) at 37°C and 5% CO₂ in humidified incubators. Media was changed every 3–4 days. The cells were passaged using a trypsin-verse solution once they had reached ~70% confluence. Cells were tested for mycoplasma contamination before treatment using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Walkersville, MD, USA). Cells were treated once they had reached 70% confluence with 1α,25-dihydroxyvitamin D₃ (from this point referred to as calcitriol; Sigma), diluted in 2400 µl 100% ethanol to make a 10 µM stock solution. The stock solution was further diluted with 100% ethanol to obtain the desired concentration for each experiment.
Controls were treated with an identical volume of ethanol for all experiments. Harvesting of cells for protein and RNA was performed after 16 h treatment, informed by results from Palmer et al.²¹ and time-course/dose-response experiments as detailed below.

### 2.2 Organoid culture, histopathological assessment and treatment

The culture of colonic organoids from human tissue has been described previously.²⁹ All participants provided informed written consent, and research was approved by local research ethics committees (13/SS/0248) and National Health Service management (2014/0058). Human colonic mucosa was removed during resectional colorectal surgery or via rectal biopsy using a rigid sigmoidoscope and rectal biopsy forceps. After washing, mucosa was incubated in crypt chelating solution (1× PBS, 45 mmol/L sucrose, 55 mmol/L D-sorbitol, 500 mmol/L Dl-dithiothreitol, 5 mmol/L EDTA) for 1 h at 4°C to dissociate the crypts. Crypts were pelleted, washed, embedded in BD Matrigel basement membrane matrix (~500 crypts per 50 µl matrigel, BD Biosciences, San Jose, CA; 356234) and maintained in human colon mucosa medium (Advanced DMEM/F12 [12634028], 1× GlutaMax [Gibco, 35050038], 1 mol/L Hepes [Gibco, 15630106], 100 IU/ml penicillin and 100 µg/ml streptomycin [Lanza, Basel, Switzerland; 09-757F], 1× B27 [Gibco, 17504044], 1 mmol/L N-acetyl-L-cysteine [Sigma-Aldrich, A7250], 10 mmol/L nicotinamide [Sigma-Aldrich, 72340], 10 mmol/L gastrin [Sigma-Aldrich, G9020], 50 ng/ml epidermal growth factor [Sigma-Aldrich, E9644], 100 ng/ml mouse Noggin [Peprotech, Rocky Hill, NJ; 250-38], 500 ng/ml A83-01 [Sigma-Aldrich, SML0788], 10 µmol/L SB202190 [Sigma-Aldrich, S7067], 100 mmol/L prostaglandin E2 [Sigma-Aldrich, P5640], 1 µg/ml human R-spondin 1 [R&D Systems, Minneapolis, MN, USA; 4645-RS], and 100 ng/ml human Wnt-3a [R&D, 5036-WN]), which was replaced every 2–3 days with passage 1:4 every 5–7 days. Selected organoid cultures were subjected to histopathological assessment using immunohistochemistry and RNA Scope for Lgr5, an intestinal stem cell marker, was performed according to the manufacturers' protocol (ACD Newark, CA, USA). Organoid cultures were treated with calcitriol or ethanol control as per cell-line experiments, with time-course and dose-response experiments performed.

### 2.3 Immunoblotting

Cell line protein extraction and western blotting for the initial dose-response experiment was performed as previously described using a rabbit monoclonal E-cadherin antibody at a concentration of 1:100 at 4°C overnight (Cell signalling, Danvers, Massachusetts, USA, 3195). The secondary antibody used was goat anti-rabbit antibody tagged with the enzyme horseradish peroxidase (at 1:1000) at room temperature for an hour and antigen-antibody complexes were visualized with chemiluminescence. β-actin was used as a loading control and was detected by a mouse monoclonal antibody at 1:4000 (Sigma), with secondary goat anti-mouse antibody at 1:1000 (Sigma).

### 2.4 RNA preparation and quantitative reverse transcription–PCR (qRT-PCR)

RNA was extracted from cells and organoids using a proprietary RNA extraction kit (Ribopure kit, Applied Biosystems, Streetsville, Ontario, Canada) according to the manufacturer's protocol. DNase treated RNA samples were then reversed transcribed to cDNA prior to qRT-PCR. Samples were added to a solution containing 1 µl (200 units) Moloney Murine Leukemia Virus Reverse Transcriptase (in 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% Nonidet® P-40 and 50% glycerol; Madison, WI, USA), 4 µl M-M:VRT buffer (50 mM Tris-HCl (pH 8.3 at 25°C; Promega), 75 mM KCl, 3 mM MgCl2 and 10 mM DTT; Promega), 2 µl dNTP (10 mM, containing dATP, dCTP, dGTP, and dTTP; Promega), 1 µl (20 units) Recombinant RNasin Ribonuclease Inhibitor (in 20 mM HEPES-KOH (pH 7.6), 50 mM KCl, 8 mM DTT, 50% (v/v) glycerol; Promega), 2 µl of 0.25 µg/µl random primers (Promega) and 1 µl RNase-free DNase-free water. This was incubated at 37°C for 30 min and 95°C for 5 min.

### 2.5 Quantitative real time polymerase chain reaction (qRT-PCR)

cDNA samples were diluted to produce a working stock (1:20 dilution for cDNA from cell lines, 1:10 from organoids). qRT-PCR was undertaken using TaqMan®
Gene Expression Assays (Applied Biosystems; CDH1, Hs01023894_m1 and Hs01023895_m1; CYP24A1, Hs00167999_m1). For each Taqman® assay, a master mix was made up using Taqman® gene expression master mix (containing AmpliTaq Gold® DNA Polymerase, Uracil-DNA glycosylase, dNTPs (with dUTP), ROX™ Passive Reference, and optimized buffer components), the Taqman expression assay and RNase-free DNase-free water with stock cDNA. Standard curves were derived from undiluted stock solutions of cDNA (HCT116 colorectal cancer cell line, untreated or treated with calcitriol) to determine the linearity and amplification efficiency of each assay. Dilution of stock cDNA was initially 1:1, with a further six 1:4 serial dilutions. Water was also included as a negative control. Threshold cycles (Ct) were plotted each assay.

Dilution of stock cDNA was initially 1:1, with a further six 1:4 serial dilutions. Water was also included as a negative control. Threshold cycles (Ct) were plotted each assay.

Dilution of stock cDNA was initially 1:1, with a further six 1:4 serial dilutions. Water was also included as a negative control. Threshold cycles (Ct) were plotted each assay.

Dilution of stock cDNA was initially 1:1, with a further six 1:4 serial dilutions. Water was also included as a negative control. Threshold cycles (Ct) were plotted each assay.

All qRT-PCR reactions were performed in triplicate, with amplification and detection of the product undertaken using the ABI PRISM® 7900HT Sequence Detection System (thermocycler; 50°C–2 min, 95°C–10 min, 95°C–15 s, 60°C–1 min repeated 40 times) and collected with SDS v2.3 software, using the FAM detector. Analysis of results included calculation against the standard curve and normalization to a reference gene derived via an online reference gene finder testing BestKeeper and NormFinder.

Standard deviation between triplicates was calculated as a measure of methodological quality.

### 2.6 Whole transcriptome profiling using HumanHT-12 v4.0 Expression BeadChip Arrays

Whole transcriptome profiling was performed on triplicate ETOH and calcitriol (50 nM) treated samples of HCT116, SW480 and LS174T cell lines and organoid samples using DNA microarray. RNA was quantified by NanoDrop (Nanodrop Technologies, Wilmington, DE, USA) and yield and integrity was assessed using the Agilent 2100 Bioanalyzer. The RNA yield and integrity of samples to be submitted for microarray expression/RNAseq analysis was assessed using the 2100 Bioanalyzer®. For HT12 analysis, total RNA was converted to double-stranded cDNA, followed by in vitro transcription amplification to generate labelled cRNA (Illumina TotalPrep™ RNA Amplification Kit). Gene expression profiling was undertaken using the HumanHT-12 v4.0 Expression BeadChip Arrays (Illumina) and iScan NO660 scanner, providing coverage of 47 231 transcripts and >31 000 annotated genes derived from the National Centre for Biotechnology Information Reference Sequence RefSeq Release 38 (November 7, 2009). Microarray data was exported from BeadStudio (Illumina) and processed in R using the limma package. In brief, the steps involved were background correction using negative controls, quantile normalization to remove technical variation and finally log2 transformation. Control probes and probes that were not expressed in at least three arrays to a detection-value of ≥5% were excluded. A standard approach to batch correction using ComBat was performed to control for batch effects.

### 2.7 RNA sequencing and analysis

Whole-genome transcriptomic patterns were analyzed on total RNA. Cell line RNA samples were subjected to 150 bp paired-end total RNA-seq (155M reads) in a single batch. RNA integrity and yield was quantified using the 2100 Bioanalyzer®. RNA samples were submitted to the Edinburgh Genomics sequencing facility, where QC, ribosomal-depletion, strand-aware library preparation and Illumina adapter ligation was performed. Ribosomal RNA was depleted using the New England Biolabs NEBNext rRNA Depletion Kit according to the manufacturer’s protocol. Samples were sequenced on the Illumina HiSeq 2500 platform in ‘rapid mode’ with 150 bp paired-end reads. Transcript indexing and quantification from RNA-seq reads was performed using Salmon v1.1.0.34 The Salmon index was generated using the reference dataset GRCh38.primary_assembly.fa and the Ensembl 96 release, (ftp://ftp.ensembl.org/pub/release-96/fasta/homo_sapiens). In brief, the Ensembl (cDNA and ncRNA) and reference FASTA were concatenated, the ‘decoys.txt’ was prepared from GRCh38 and the Salmon index run on the concatenated FASTA file in the R statistical environment (v4.0.0). Paired reads 1 and read 2 fastq files respectively were then concatenated for each sample, and Salmon run using auto-detect strandedness and ‘validateMappings’ flag.

Salmon transcript level quantitation files were combined and summarized to gene-level counts using tximport and biomaRt.36,37 Transcript level counts were aggregated into gene levels counts and imported into R using the tximport package. A Differential Gene Expression object (DGEList) was created using tximport generated gene-level counts and sample metadata for analysis using edgeR (3.30.0) and limma (3.44.1). Genes were filtered using the filterByExpr function in the edgeR package (3.30.0) to include genes with at least 10 reads per gene. The calcNormFactors function was used to calculate Trimmed mean of M-values (TMM) between-sample normalization to scale the library size. The limma ‘voom’ function was used to transform normalized count data.
to log2-counts per million (logCPM), estimate the mean-variance relationship and used to compute appropriate observation-level weights.

2.8 | Genotype at the vitamin D pathway loci

Cell line genomic DNA was genotyped for rs2282679 known to be associated with 25-OHD level.38 Three functionally relevant polymorphisms in the VDR gene which are established as functionally relevant, impacting VDR function, DNA binding and calcitriol binding (rs1544410, rs10735810, rs7975232) were also genotyped by using the Infinium Omni5 BeadChip (Illumina, San Diego, CA, USA).

2.9 | Mass-spectrometry

Cell line samples were prepared for mass-spectrometry analysis using a Guanidinium lysis followed by LysC and Trypsin digest as described.39 Briefly, the cells were lysed in 50 µl 6M GuHCl, 100 mM Tris pH 8.5 with 1 mg/ml Chloracetamide and 1.5 mg/ml TCEP.

The cells were probe sonicated until lysed then heated to 95°C for 5 min. and digested with 1/200 (Enzyme to Substrate) LysC (Wako Chemicals, Japan) for 4 h at 37°C. Samples were diluted with 250 µl containing 1/200 (Enzyme to Substrate) porcine Trypsin (Promega, UK) and the digest was continued overnight. Peptides samples were acidified with TFA to 0.5% (final concentration) and cleared by centrifugation for 10 min on a benchtop centrifuge. Samples were desalted using Stage-tips as described.40 Mass spectrometry was performed using a Lumos Fusion (Thermo) mass spectrometer coupled to a RLS-nano uHPLC (Thermo). Peptides were separated by a 140 min linear gradient from 5% to 30% acetonitrile, 0.05% acetic acid. Proteins were identified and quantified the MaxQuant software suite41 using label-free quantification and searching against the Human Uniprot database.

2.10 | Statistical analysis

All statistical analysis was undertaken in R.42 Investigation of differential gene expression in response to calcitriol treatment was undertaken using the lmFit and eBayes functions within the ‘limma’ package.43 Differentially expressed gene-sets were assessed for enrichment across expression analysis methodologies and between in vitro and ex vivo datasets using the geneSetTest function in R. Ranked gene lists were assessed for functional relevance using the ‘GOrilla’, Gene Ontology enRICHment analyLysis and visualizAtion tool.44 Process ontologies were validated by Gene Set Enrichment Analysis using the gseGO function within the ‘clusterprofiler’ package in R. 45

3 | RESULTS

3.1 | Calcitriol treatment induces CDH1 and E-cadherin expression in colorectal cancer cell lines

We optimized treatment conditions, using SW480 cells with CYP24A1 and CDH1 expression as readouts. CYP24A1, which encodes 24-hydroxylase a key enzyme in vitamin D metabolism, was chosen as positive control, while CDH1, a tumor suppressor gene which encodes E-cadherin, was assessed given reported induction in SW480 cells by calcitriol.21 Significant induction of both CYP24A1 (fold-change [FC] = 4.45 95% CI 3.00–5.89) and CDH1 (FC = 3.60 95% CI 2.17–5.03) was observed with 50 nM calcitriol treatment over 16-h, adopted hereafter as standard treatment conditions (Figures S1 and 1).

Next, we tested for CDH1 induction in a panel of cell lines. The 10 cell lines treated represent CRC as a whole, given the broad tumors of origin (see Supplementary Methods) and are known to differ in their mutation profile e.g., microsatellite instability, CpG island methylator phenotype status and molecular subtype.46 Significant induction of CDH1 by calcitriol treatment was observed in SW480, HCT116 and LS174T cell lines, with induction of E-Cadherin seen in SW480 and HCT116 cells but not LS174T (Figure 1). No association between cell genotype at vitamin D pathway SNPs (rs2282679, rs1544410, rs10735810, rs7975232) and either baseline CDH1, or induction with calcitriol was observed (p > .05).

3.2 | Calcitriol influences expression of 30 genes in CRC cell lines

We chose three cell lines for HT12 microarray analysis based on CDH1 induction on qRT-PCR (SW480, HCT116, LS174T). Analysis confirmed the induction of both CDH1 and CYP24A1 by 50 nM calcitriol when results were pooled for the three cell lines (CDH1 logFC = 0.80, FDR = 0.0001, CYP24A1 logFC = 3.70 FDR = 0.02; Figure S2), demonstrating strong correlation with qRT-PCR expression data: (CDH1 r = 0.91, p < 2.2e−16; CYP24A1 r = 0.88, p < 2.2e−16). In total, 30 genes were differentially expressed after calcitriol treatment (FDR < 0.05, Table S1). When considered separately, there were 22, 972 and 417 significantly differentially
FIGURE 1  (A) Replicate expression fold-change of CYP24A1 and CDH1 in SW480 cells with 16 h 500 pM–50 nM. Cells were treated with 500 pM or 50 nM calcitriol or equivalent volume of ETOH for 16 h and harvested for RNA. Up to 11 biological replicate experiments were performed. qRT-PCR was performed to assess expression of CYP24A1 and CDH1 normalized to ACTB and average fold-change expression relative to ETOH control was charted. Student’s t-test was performed on fold-changes per treatment. *p < .05, **p < .01, ***p < .001. Error bars represent standard error of mean (SEM).  (B) Induction of CDH1 by calcitriol in panel of CRC cell lines. 10 cell lines were treated in triplicate with 50 nM calcitriol or equivalent volume of ETOH for 16 h and harvested for RNA. qRT-PCR was performed to assess expression of CDH1 normalized to ACTB and average fold-change in expression relative to ETOH control was charted. Error bars represent SEM of biological replicate samples. Student’s t-test was performed on fold-changes per treatment. *p < .05, **p < .01, ***p < .001.  (C) Western blot of E-cadherin expression in ETOH treated cell lines. Cells were treated for 16 h ETOH and harvested for protein. Western blotting was performed with a commercial E-cadherin antibody (Cell Signalling, #3195), with β-actin (Sigma, #A1978) as loading control.  (D) Western blot of E-cadherin expression with 50 nM calcitriol treatment. Cells were treated for 16 h with 50 nM calcitriol or ETOH and harvested for protein. Western blotting was performed with a commercial E-cadherin antibody (Cell Signalling, #3195), with β-actin (Sigma, #A1978) as loading control.
expressed genes HCT116, LS174T and SW480 cell samples respectively. We sought technical replication of our results using RNA-sequencing. In total, 59 named genes were differentially expressed (Table S2), with CDH1 the fourth hit (logFC=0.85, FDR = 0.002, Figure S3) and strong correlation between expression values for differentially expressed genes between HT12/RNAseq ($r = 0.85$, $p = 1.8e-08$, Figure S4). Significant enrichment of the 30 differentially expressed genes on HT12 in the RNA-seq dataset was observed ($2.39e-10$, Figure S5).

3.3 GO term enrichment indicates dysregulation of anti-tumor effects with calcitriol

Functional annotation of the HT12 gene list identified 63 significantly enriched ontologies, with the top 10 and additional relevant terms listed in Table 1. Results from GOrilla analysis were replicated using GSEA, which identified 131 significantly enriched terms and confirmed enrichment of 20 common processes seen with GOrilla, as depicted in Figure S6. A number of additional terms identified by GSEA had links to anti-tumor processes, e.g., ‘regulation of Wnt signaling pathway’, ‘columnar/cuboidal epithelial cell differentiation’ and ‘cell adhesion’ (Table S3). GOrilla and GSEA analysis were repeated using the ranked list from RNA-seq analysis. 127 significantly enriched terms were identified using GOrilla (Table S4), with 21 terms in common with analysis of the HT12 dataset and notable terms including ‘regulation of cell migration’ and ‘negative regulation of cell adhesion’. 65 terms were enriched when using GSEA (Table S5), with 4 common terms with HT12 analysis including ‘biological adhesion’, ‘cell surface receptor signaling pathway’ and ‘cell adhesion’.

3.3.1 Mass spectrometry and the effect of calcitriol on cell line proteome

Mass spectrometry detected a large number of proteins across the samples tested (HCT116 $n = 3880$, LS174T

| Description                                      | FDR       | Enrichment | Number of genes |
|--------------------------------------------------|-----------|------------|-----------------|
| Anatomical structure morphogenesis                | 3.28e-04  | 2.15       | 67              |
| Regulation of multicellular organismal process    | 1.38e-03  | 1.38       | 251             |
| Regulation of body fluid levels                   | 1.06e-03  | 2.14       | 55              |
| Developmental process                             | 1.84e-03  | 1.31       | 312             |
| Anatomical structure development                  | 1.49e-03  | 1.64       | 110             |
| Regulation of epithelial cell proliferation       | 5.03e-03  | 3.16       | 25              |
| Placenta development                              | 8.87e-03  | 59.03      | 4               |
| Negative regulation of cell proliferation        | 9.28e-03  | 2.13       | 48              |
| Regulation of multicellular organismal development| 8.79e-03  | 1.78       | 76              |
| Anatomical structure morphogenesis                | 3.28e-04  | 2.15       | 67              |
| Additional relevant significantly enriched terms   |           |            |                 |
| Regulation of cell differentiation (19)           | 1.66e-02  | 1.43       | 154             |
| Regulation of cell proliferation (33)             | 2.47e-02  | 1.66       | 79              |
| Negative regulation of cell differentiation (42)  | 3.99e-02  | 1.64       | 72              |
| Regulation of epithelial cell differentiation (71)| 5.79e-02  | 3.59       | 14              |
| Regulation of cell migration (116)                | 8.44e-02  | 1.87       | 40              |

Note: HCT116, LS174T and SW480 cell lines were treated in triplicate with 50 nM calcitriol or ETOH and gene expression in harvested RNA assessed using HT12 microarray. Differential expression was calculated using Student’s t-test and genes ranked by significance entered into the GO enrichment software GOrilla. FDR q-value is the correction of the above p-value for multiple testing using the Benjamini and Hochberg method. For the ith term (ranked according to p-value) the FDR q-value is (p-value * number of GO terms)/i. Enrichment = (the number of genes in the intersection/number of genes in the top of the input list)/(total number of genes associated with a specific GO term/total number of genes). Additional relevant processes not ranking in top 10 are given (rank).
\( n = 3889, \text{SW480} \; n = 3845 \). Increased expression of 1,25OHD 24-hydroxylase, encoded by CYP24A1, was seen across all cell lines \((p < .0005)\) and the top ten significantly altered proteins in each cell line are given in Table S6. On pooling cell line data, no significantly altered proteins were identified after adjustment for multiple testing, yet enrichment analysis of this ranked list using REACTOME V57 revealed 216 significant entities \((\text{FDR} < 0.05)\), with a number of pathways linked to tumorigenesis, e.g., ‘Cell cycle’ and ‘Apoptosis’ \((\text{Table S7 and Figure S7})\).

### 3.4 | Calcitriol treatment induces ex vivo \(CDH1\) and E-cadherin expression

We used patient-derived colorectal organoids to validate the effect of calcitriol on epithelial cell gene expression and processes relevant to tumorigenesis \((\text{Figures 2 and S8})\). Time-course and dose-response experiment demonstrated induction of \(\text{CYP24A1 and CDH1 after 50–100 nM} conspiracy treatment, validating in vitro observations of both calcitriol metabolism and \(\text{CDH1 induction. Calcitriol treatment (100 nM, 24 h)}\) of 6 further patient-derived organoid cultures \((\text{Figures S1 and 2})\), consistently induced both \(\text{CYP24A1 and CDH1 expression.}\)

### 3.5 | Calcitriol influences expression of 111 genes in patient-derived organoids

RNA from paired calcitriol treated/untreated non-FAP organoids \((\text{patient ID B, C & D})\) was submitted for microarray analysis. This identified 111 differentially expressed genes \((\text{top hit} \; CD14, \log \text{FC} = 4.91, \text{FDR 4.47e−05, Table S8})\). The differentially expressed gene-set from cell line data was significantly enriched \((p = 3.97e−08)\) in the organoid dataset indicating conservation of gene expression responses to calcitriol across these two model systems, with six individual genes differentially expressed \((\text{FDR} < 0.05)\) in both cell line and organoid samples \((\text{Figure 3})\). These included \(\text{EFTUD1 (FC = 1.59 in vitro, FC = 3.26 ex vivo)}\); and \(\text{GADD45A (FC = 1.53 in vitro, FC = 2.45 ex vivo)}\), which were induced and \(\text{KIAA1199 (FC = 0.71 in vitro, FC = 0.25 ex vivo)}\), which was suppressed \((\text{Tables S9 and S10})\). GO enrichment \((\text{GORilla})\) revealed 328 significantly enriched terms, with many relevant to tumorigenesis, including ‘cell migration’, ‘regulation of cell adhesion’, ‘regulation of cell death’ and ‘regulation of cell differentiation’ \((\text{Table S11})\). Seventeen GO terms were enriched \((\text{FDR} \; p < .05)\) in both cell line and organoid samples, including many relevant to tumorigenesis \((\text{e.g., ‘regulation of cell proliferation’, ‘regulation of cell differentiation’, selected relevant terms Figure 3})\). Replication of enrichment analysis using GSEA confirmed enrichment of similar terms \((\text{e.g., ‘epithelial cell differentiation’, ‘apoptotic signalling pathway’, ‘regulation of cell death’})\) and identified 38 confirmed common terms with in vitro dataset.

### 4 | DISCUSSION

We report for the first-time, common genes and pathways modulated by calcitriol treatment across both in vitro \((\text{cell lines})\) and ex vivo \((\text{organoids})\) models including \(\text{CDH1}\) and several other genes linked to tumorigenesis. The enrichment of pathways relating to cell differentiation, proliferation, migration and death in addition to immune processes support epidemiological data of the role of vitamin D as a chemopreventive agent. These data provide strong rationale for further well-designed trials of vitamin D supplementation for CRC prevention.

The \(\text{CDH1 gene, codes for E-cadherin, a glycoprotein required for effective cell–cell adhesion. E-cadherin also regulates β-catenin signalling in the Wnt pathway, the aberrant activation of which has been shown to be an initiating event in the development of CRC.}\) The loss of E-cadherin expression is also central to the epithelial-to-mesenchymal transition, a key step in the metastatic progression of human cancers, and reduced \(\text{CDH1 expression through mutation or promoter methylation is seen in several epithelial cancers, resulting in increased invasive-ness and a reduced survival in vivo. Inactivating germline mutations are reported in familial aggregations of gastric, colonic and breast cancers. Here we show induction of \(\text{CDH1 in several established CRC cell lines and in organoids derived from normal mucosa sampled from various sites along the colorectum. Our study expands on work by Palmer et al., who reported an induction of E-Cadherin in SW480 colorectal cancer cells treated with 1,25OHD.}^2\)

Here, we identify additional CRC cell lines with disparate molecular characteristics and mutation profile in which \(\text{CDH1 is induced by 1,25OHD, providing a potential mechanism to underlie risk/survival associations between vitamin D status/supplementation and colorectal cancer.}\) Transcriptomic analysis identifies a number of other genes which are modulated by calcitriol treatment. These genes provide targets for validation and future mechanistic investigation. Within the common gene-set both \(\text{SULT1C2}^{20}\) and \(\text{CYP24A1}\) are known to be induced by calcitriol providing confidence that calcitriol is activating the vitamin D pathway in both culture models. Of particular interest is the consistent upregulation of \(\text{GADD45A}\) by calcitriol. The Growth Arrest and DNA Damage-inducible 45 \((\text{GADD45})\) proteins have been implicated in regulation of many cellular functions including DNA repair, cell cycle control, senescence and genotoxic stress.
FIGURE 2  (A) Microscope image of patient-derived colonic organoids: Colonic organoids were derived from stripped colonic. Following culture medium change on days 3 and 5, photographs were taken on day 8 using the Nikon TiS Tissue Culture microscope (×4 magnification). These images show the characteristic spherical organoids. (B) Representative images of histopathological assessment of human normal mucosa intestinal organoids; top left- H&E stain of paraffin embedded normal mucosa organoids; top right- RNA in situ hybridization (RNAscope™) of intestinal stem cell marker LGR5, cyan dots show LGR5 transcripts; bottom left- immunohistochemistry of epithelial marker β-catenin; bottom right- immunohistochemistry of proliferative marker Ki-67. (C) Clinical and culture characteristics of patient-derived organoids. (D) Induction of CDH1 in patient-derived colorectal organoids. Human intestinal organoids were derived from normal mucosa from six patients and treated with calcitriol. Organoids from six wells were pooled for each RNA sample, and qRT-PCR used to assess gene expression. Error bars represent SEM of biological replicates. Student’s t-test was performed on fold-changes per treatment. **p < .01, ***p < .001
Furthermore, aberrations in the GADD45 pathway and dysregulation of GADD45 expression is associated with the initiation and progression of malignancies and inhibition of GADD45 through promoter methylation or NF-κB activation, and is considered a critical step in cancer development. Meanwhile, induction of GADD45 expression is reported to be an essential step for mediating anti-cancer activity of multiple chemotherapeutic drugs. Induction of GADD45 has previously been reported in prostate, ovarian, squamous and colorectal cancer cell lines supporting our current findings. Similarly, EFTUD1 is also known to be upregulated by calcitriol in breast cancer and breast tumor explants and elevated EFTUD1 predicts prolonged breast cancer survival. Finally, KIAA1199, which is downregulated by calcitriol in our study, is a known oncogene which plays a role in proliferation, apoptosis, invasion and migration of various tumor cells. It is overexpressed in colorectal cancer with higher levels associated with increased tumor invasion depth, stage, and poor prognosis in CRC. To the best of our knowledge this is the first study to report suppression of intestinal epithelial cell KIAA1199 expression with calcitriol, providing an exciting target for further study.

Our ex vivo dataset adds to a small number of studies demonstrating gene expression changes in patient-derived organoids treated with vitamin D.
Fernández-Barral et al., demonstrated upregulation of stemness-related genes and inhibition of cell proliferation in patient-derived colonic organoids, with differential expression of >2000 genes. This gene-set was significantly expressed after calcitriol treatment of our organoids ($p = 2.496e-93$; Figure S9). The intersect of common differentially expressed genes was 66, including GADD45A, with complete concordance in direction of modulation (i.e., up- or down-regulated). Enrichment of our recently reported gene-set associated with 25-OH level in human rectal mucosa was also seen in our organoid data, providing further validation of our results ($p = .0075$, Figure S9).

Pathway analysis in the current study confirms implied links with carcinogenesis at individual gene level. Such terms as ‘cell migration’, ‘regulation of cell adhesion’, ‘regulation of cell death’, ‘regulation of cell differentiation’ and ‘cell cycle’ support numerous previous in vitro studies demonstrating vitamin D-induced growth arrest and apoptosis of colorectal cancer cells, modulation of the Wnt signalling pathway, DNA repair and immunomodulation,\(^5\) thus lending support to a causal relationship between vitamin D and cancer. Several clinical studies have replicated these in vitro findings in vivo—Protiva et al., reported upregulation of genes involved in inflammation, immune response, extracellular matrix, and cell adhesion in response to 1,25OHD,\(^6\) while a randomized study of vitamin D3 supplementation reported a reduction in tumor promoting inflammation biomarkers, decreased oxidative DNA damage, increased cell differentiation and apoptosis and modification of the APC/β-catenin pathway in the normal human colorectal epithelium.\(^61–65\) Thus, our current findings confirm further possible mechanisms to underlie the observed association between vitamin D deficiency and increased CRC risk.

Whilst the complementary in vitro/ex vivo design of our study has many positive attributes, we acknowledge several limitations. First, we acknowledge the limitations of comparative cell line studies including the risk that accumulated cellular genomic and karyotypic abnormalities may significantly influence results from experimental studies. However, the replication of several common genes and pathways in our ex vivo studies, i.e., a non-aberrant mode system, provides confidence in our results. Next, for statistical power, we pooled the different cell lines and organoid for transcriptomic profiling/sequencing which ignores potential differential responses between cultures and cell lines. However, even despite this, and the known heterogeneity in gene expression between cell lines or colorectal epithelial cell sampling site,\(^66\) relevant differential gene expression and pathway enrichment is observed, indicating conservation of responses to vitamin D irrespective of cell line differences or sampling site, thus supporting the broad potential of vitamin D as valuable adjunctive chemotherapeutic agent in CRC. We acknowledge that the smaller number of differentially expressed genes in the in vitro studies likely reflects the different characteristics of the cell lines studied, confirmed by the marked difference in significantly differentially expressed genes between the three cell lines. Further detailed investigation of the relevance of these differences in respect of baseline cell line characteristics and mechanistic studies to further define the importance of potential vitamin D target genes identified here are required. Finally, we acknowledge that RNA-sequencing is now the predominant method of transcriptome gene expression analysis. HT12 analysis was used as our primary analysis platform for the exploratory analyses providing data on >31 000 annotated genes as the most practical and cost-effective method of transcriptomic profiling. We replicated the induction of CDH1 at a technical level (qRT-PCR, HT12, RNA-seq), while the modulation of other genes of interest (GADD45, KIAA1199, GADD45A) was conserved across both microarray and RNA-seq platforms. Other groups have published reports of good high levels of reproducibility between sequencing and microarray platforms.\(^67\) Even if correlation at a transcript/probe level was not observed, RNA-seq and microarray can be seen to be complementary.\(^68\) As such, the differences in platform and methodology that we have utilized, with reassuring agreement in differential expression and gene-set enrichment with RNAseq/HT12 data provides confidence that these are true effects.

In conclusion, we show that calcitriol induces induction of CDH1, GADD45 and EFTUD1 in both CRC cell lines and patient-derived epithelial cell organoids, with suppression of KIAA1199 also observed; genes with recognized links to CRC tumorigenesis. Furthermore, several pathways modulated by calcitriol treatment are intimately linked to tumorigenesis. These findings support epidemiological data and provide further strong rationale for well-designed trials of vitamin D supplementation as a novel CRC chemopreventive and chemotherapeutic agent.

**ACKNOWLEDGEMENTS**

We acknowledge the excellent technical support from Marion Walker, Stuart Reid and Asta Valanciute. We are grateful to Donna Markie and Fiona McIntosh, and all those who continue to contribute to our translational programme. We acknowledge the expert support on sample preparation from the Genetics Core of the Edinburgh Wellcome Trust Clinical Research Facility.
DISCLOSURES
The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS
Conceptualization, Peter G. Vaughan-Shaw, Susan M. Farrington, Malcolm G. Dunlop; Methodology, Peter G. Vaughan-Shaw, Li-Yin Ooi, Farhat V. N. Din, Alex von Kriegsheim, Susan M. Farrington, Malcolm G. Dunlop; Investigation, Peter G. Vaughan-Shaw, Li-Yin Ooi, James P. Blackmur, Anna M. Ochocka-Fox, Alex von Kriegsheim, Graeme Grimes, Maria Timofeeva, Marion Walker, Victoria Svinti, Vidya Rajasekaran, Karen Dunbar; Writing Original Draft Peter G. Vaughan-Shaw, Susan M. Farrington, Malcolm G. Dunlop; Writing – Review & Editing, James P. Blackmur, Maria Timofeeva, Susan M. Farrington, Farhat V. N. Din, Malcolm G. Dunlop; Funding Acquisition, Malcolm G. Dunlop; Resources, Malcolm G. Dunlop; Supervision, Susan M. Farrington and Malcolm G. Dunlop.

DATA AVAILABILITY STATEMENT
The data generated in this study are publicly available in Gene Expression Omnibus (GEO) at GSE181644.

ORCID
Peter G. Vaughan-Shaw https://orcid.org/0000-0002-9790-6882
Susan M. Farrington https://orcid.org/0000-0001-5955-7389

REFERENCES
1. Theodoratou E, Tzoulaki I, Zgaga L, Ioannidis JPA. Vitamin D and multiple health outcomes: umbrella review of systematic reviews and meta-analyses of observational studies and randomised trials. BMJ. 2014;348:g2035.
2. Autier P, Boniol M, Pizot C, Mullie P. Vitamin D status and ill health: a systematic review. Lancet Diabetes Endocrinol. 2014;2(1):76-89. doi:10.1016/S2213-8587(13)70165-7
3. He Y, Timofeeva M, Farrington SM, et al. Exploring causality in the association between circulating 25-hydroxyvitamin D and colorectal cancer risk: a large Mendelian randomisation study. BMC Med. 2018;16(1):142. doi:10.1186/s12116-018-1119-2
4. Meng X, Li X, Timofeeva MN, et al. Phenome-wide Mendelian-randomization study of genetically determined vitamin D on multiple health outcomes using the UK Biobank study. Int J Epidemiol. 2019;48(5):1425-1434. doi:10.1093/ije/dyz2182
5. Ye Y, Yang H, Wang Y, et al. A comprehensive genetic and epidemiological association analysis of vitamin D with common diseases/traits in the UK Biobank. Genet Epidemiol. 2021;45(1):24-35. doi:10.1002/gepi.22357
6. Manson JE, Cook NR, Lee I-M, et al. Vitamin D supplements and prevention of cancer and cardiovascular disease. N Engl J Med. 2019;380(1):33-44. doi:10.1056/NEJMoa1809944
7. Scragg R, Khaw K-T, Toop L, et al. Monthly high-dose vitamin D supplementation and cancer risk: a post hoc analysis of the vitamin D assessment randomized clinical trial. JAMA Oncol. 2018;4(11):e182178. doi:10.1001/jamaoncol.2018.2178
8. Barry EL, Peacock JL, Rees JR, et al. Vitamin D receptor genotype, vitamin D3 supplementation, and risk of colorectal adenomas. JAMA Oncol. 2017;3(5):628. doi:10.1001/jamaoncol.2016.5917
9. Hermann J, Eder P, Banasiewicz T, Matusiak K, Lykowska-Szuber L. Current management of anal fistulas in Crohn’s disease. Prz Gastroenterol. 2015;10(2):83-88. doi:10.5114/pg.2015.49684
10. Vaughan-Shaw PG, O’Sullivan F, Farrington SM, et al. The impact of vitamin D pathway genetic variation and circulating 25-hydroxyvitamin D on cancer outcome: systematic review and meta-analysis. Br J Cancer. 2017;116(8):1092-1110. doi:10.1038/bjc.2017.44
11. Vaughan-Shaw PG, Zgaga L, Ooi LY, et al. Low plasma vitamin D is associated with adverse colorectal cancer survival after surgical resection, independent of systemic inflammatory response. Gut. 2020;69(1):103-111. doi:10.1136/gutjnl-2018-317922
12. Keum N, Lee DH, Greenwood DC, Manson J, Giovannucci E. Vitamin D supplementation and total cancer incidence and mortality: a meta-analysis of randomized controlled trials. Ann Oncol. 2019;30(5):733-743. doi:10.1093/annonc/mdz059
13. Vaughan-Shaw PG, Bijsis LF, Blackmur JP, et al. The effect of vitamin D supplementation on survival in patients with colorectal cancer: systematic review and meta-analysis of randomised controlled trials. Br J Cancer. 2020;123(11):1705-1712. doi:10.1038/s41416-020-01060-8
14. Dou R, Ng K, Giovannucci EL, et al. Vitamin D and colorectal cancer: molecular, epidemiological and clinical evidence. Br J Nutr. 2016;115(9):1643-1660. doi:10.1017/S0007114516006969
15. Bertucci F, Salas S, Eysteries S, et al. Gene expression profiling of colon cancer by DNA microarrays and correlation with histoclinical parameters. Oncogene. 2004;23(7):1377-1391. doi:10.1038/sj.onc.1207262
16. Chan SK, Griffith OL, Tai IT, et al. Meta-analysis of colorectal cancer gene expression profiling studies identifies consistently reported candidate biomarkers. Cancer Epidemiol Biomark Prev. 2008;17(3):543-552. doi:10.1158/1055-9965.Epi-07-2615
17. Birkenkamp-Demtroder K, Christensen LL, Olesen SH, et al. Gene expression in colorectal cancer. Can Res. 2002;62(15):4352-4363.
18. Pike JW, Meyer MB. The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D3. Endocrinol Metab Clin North Am. 2010;39(2):255-269. doi:10.1016/j.ecl.2010.02.007
19. Ramagopalan SV, Heger A, Berlanga AJ, et al. A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. Genome Res. 2010;20(10):1352-1360. doi:10.1101/gr.107920.110
20. Wood RJ, Tchack L, Angelo G, et al. DNA microarray analysis of vitamin D-induced gene expression in a human colon carcinoma cell line. Physiol Genomics. 2004;17(2):122-129. doi:10.1152/physiogenomics.00002.2003
21. Palmér HG, González-Sancho JM, Espada J, et al. Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling. J Cell Biol. 2001;154(2):369-388. doi:10.1083/jcb.200102028
22. Yu X, Wang Q, Liu B, Zhang N, Cheng G. Vitamin D enhances radiosensitivity of colorectal cancer by
reversing epithelial-mesenchymal transition. *Front Cell Dev Biol*. 2021;9:684855. doi:10.3389/fcell.2021.684855

23. Swami S, Raghavachari N, Muller UR, Bao YP, Feldman D. Vitamin D growth inhibition of breast cancer cells: gene expression patterns assessed by cDNA microarray. *Breast Cancer Res Treat*. 2003;80(1):49–62. doi:10.1023/A:1024487118457

24. Krishnan AV, Peelh DM, Feldman D. Inhibition of prostate cancer growth by vitamin D: regulation of target gene expression. *J Cell Biochem*. 2003;88(2):363-371. doi:10.1002/jcb.10334

25. Costales-Carrera A, Fernandez-Barral A, Bustamante-Madrid P, et al. Comparative study of organoids from patient-derived normal and tumor colon and rectal tissue. *Cancers*. 2020;12(8):2302. doi:10.3390/cancers12082302

26. Fernandez-Barral A, Costales-Carrera A, Buira SP, et al. Vitamin D differentially regulates colon stem cells in patient-derived normal and tumor organoids. *FEBS J*. 2020;287(1):53-72. doi:10.1111/febs.14998

27. McCray T, Pacheco JV, Loitz CC, et al. Vitamin D sufficiency enhances differentiation of patient-derived prostate epithelial organoids. *iScience*. 2021;24(1):101974. doi: 10.1016/j.isci.2020.101974

28. UKCCCR. UKCCCR guidelines for the use of cell lines in cancer research. *Br J Cancer*. 2000;82(9):1495-1509. doi: 10.1054/bjoc.1999.1169

29. Sato T, Stange DE, Ferrante M, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology*. 2011;141(5):1762-1772. doi:10.1053/j.gastro.2011.07.050

30. Din FV, Valancute A, Houde VP, et al. Aspirin inhibits mTOR signaling, activates AMP-activated protein kinase, and induces autophagy in colorectal cancer cells. *Gastroenterology*. 2012;142(7):1504-1515.e3. doi:10.1053/j.gastro.2012.02.050

31. Pfaffl MW, Tichopad A, Prgomet C, et al. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pairwise correlations. *Biotechnol Lett*. 2004;26(6):509-515.

32. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*. 2004;64(15):5245-5250. doi:10.1158/0008-5472.can-04-0496

33. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics*. 2007;8(1):118-127. doi:10.1093/biostatistics/kxj037

34. Patro R, Duggal G, Love MI, et al. Salmon provides fast and bias-corrected sample integrity: BestKeeper–Excel-based tool using pairwise correlations. *Biotechnol Lett*. 2004;26(6):509-515.

35. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics*. 2007;8(1):118-127. doi:10.1093/biostatistics/kxj037

36. Durinck S, Spellman PT, Birney E, et al. Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. *Nat Protoc*. 2009;4(8):1184-1191. doi:10.1038/nprot.2009.97

37. Durinck S, Moreau Y, Kasprzyk A, et al. BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. *Bioinformatics*. 2005;21(16):3439-3440. doi:10.1093/bioinformatics/bti525

38. Wang TJ, Zhang F, Richards JB, et al. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet*. 2010;376(9736):180-188. doi:10.1016/S0140-6736(10)60588-0

39. Jessie-Christensen RR, Sultan A, Olsen JV. Simple and reproducible sample preparation for single-shot phosphoproteomics with high sensitivity. *Methods Mol Biol*. 2016;1355:251-260. doi:10.1007/978-1-4939-3049-4_17

40. Turriziani B, Garcia-Munoz A, Pilkington R, et al. On-beads digestion in conjunction with data-dependent mass spectrometry: a shortcut to quantitative and dynamic interaction proteomics. *Biology*. 2014;3(2):320-332. doi:10.3390/biology3020320

41. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol*. 2008;26(12):1367-1372. doi:10.1038/nbt.1511

42. R Core Team. *R: A Language and Environment for Statistical Computing* [Program]. R Foundation for Statistical Computing; 2018.

43. Ritchie ME, Shipman B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43(7):e47. doi:10.1093/nar/gkv007

44. Eden E, Navon R, Steinfeld I, et al. GOriilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics*. 2009;10:48. doi:10.1186/1471-2105-10-48

45. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005;102(43):15545-15550. doi:10.1073/pnas.0506580102

46. Berg KCG, Eide PW, Eilertsen IA, et al. Multi-omics of 34 colorectal cancer cell lines—a resource for biomedical studies. *Mol Cancer*. 2017;16(1):116. doi:10.1186/s12943-017-0691-y

47. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B*. 1995;57(1):289-300. doi:10.1111/j.2517-6161.1995.tb02031.x

48. Hauw R, Hermjakob H, D'Eustachio P, et al. Reactome pathway analysis to enrich biological discovery in proteomics data sets. *Proteomics*. 2011;11(18):3598-3613. doi:10.1002/pmc.20110066

49. Houlston RS, Webb E, Broderick P, et al. Meta-analysis of genome-wide association data identifies four new susceptibility loci for colorectal cancer. *Nat Genet*. 2008;40(12):1426-1435. doi:10.1038/ng.262

50. Rondini EA, Fang H, Runge-Morris M, et al. Regulation of human cytotoxic sulfotransferases 1C2 and 1C3 by nuclear signaling pathways in LS180 colorectal adenocarcinoma cells. *Drug Metab Dispos*. 2014;42(3):361-368. doi:10.1124/dmd.113.055673

51. Tamura RE, de Vasconcellos JF, Sarkar D, et al. GADD45 proteins: central players in tumorigenesis. *Curr Mol Med*. 2012;12(5):634-651. doi:10.2174/156652412800619978

52. Flores O, Burnstein KL. GADD45gamma: a new vitamin D-regulated gene that is antiproliferative in prostate cancer cells. *Endocrinology*. 2010;151(10):4654-4664. doi:10.1210/en.2010-0434

53. Jiang F, Li P, Fornace AJ Jr, et al. G2/M arrest by 1,25-dihydroxyvitamin D3 in ovarian cancer cells mediated through the induction of GADD45 via an exonic enhancer.
54. Akutsu N, Lin R, Bastien Y, et al. Regulation of gene expression by 1alpha,25-dihydroxyvitamin D3 and its analog EB1089 under growth-inhibitory conditions in squamous carcinoma cells. Mol Endocrinol. 2001;15(7):1127-1139. doi:10.1210/mend.15.7.0655

55. Palmer HG, Sanchez-Carbayo M, Ordonez-Moran P, et al. Genetic signatures of differentiation induced by 1alpha,25-dihydroxyvitamin D3 in human colon cancer cells. Cancer Res. 2003;63(22):7799-7806.

56. Sheng L, Anderson PH, Turner AG, et al. Identification of vitamin D3 target genes in human breast cancer tissue. J Steroid Biochem Mol Biol. 2016;164:90-97. doi:10.1016/j.jsbmb.2015.10.012

57. Liu J, Yan W, Han P, et al. The emerging role of KIAA1199 in cancer development and therapy. Biomed Pharmacother. 2021;138:111507. doi:10.1016/j.biopha.2021.111507

58. Vaughan-Shaw PG, Grimes G, Blackmur JP, et al. Oral vitamin D supplementation induces transcriptomic changes in rectal mucosa that are linked to anti-tumour effects. BMC Med. 2021;19(1):174. doi:10.1186/s12916-021-02044-y

59. Fleet JC, DeSmet M, Johnson R, et al. Vitamin D and cancer: a review of molecular mechanisms. Biochem J. 2012;441:61-76. doi:10.1042/Bj20110744

60. Protiva P, Pendyala S, Nelson C, et al. Calcium and 1,25-dihydroxyvitamin D3 modulate genes of immune and inflammatory pathways in the human colon: a human crossover trial. Am J Clin Nutr. 2016;103(5):1224-1231. doi:10.3945/ajcn.114.105304

61. Fedirko V, Bostick RM, Flanders WD, et al. Effects of vitamin D and calcium supplementation on markers of apoptosis in normal colon mucosa: a randomized, double-blind, placebo-controlled clinical trial. Cancer Prev Res (Phila). 2009;2(3):213-223. doi:10.1158/1940-6207.CAPR-08-0157

62. Fedirko V, Bostick RM, Long Q, et al. Effects of supplemental vitamin D and calcium on oxidative DNA damage marker in normal colorectal mucosa: a randomized clinical trial. Cancer Epidemiol Biomarkers Prev. 2010;19(1):280-291. doi:10.1158/1055-9965.EPI-09-0448

63. Fedirko V, Bostick RM, Flanders WD, et al. Effects of vitamin D and calcium on proliferation and differentiation in normal colon mucosa: a randomized clinical trial. Cancer Epidemiol Biomarkers Prev. 2009;18(11):2933-2941. doi:10.1158/1055-9965.EPI-09-0239

64. Ahearn TU, Shaukat A, Flanders WD, Rutherford RE, Bostick RM. A randomized clinical trial of the effects of supplemental calcium and vitamin D3 on the APC/β-catenin pathway in the normal mucosa of colorectal adenoma patients. Cancer Prev Res (Phila). 2012;5(10):1247-1256. doi:10.1158/1940-6207.capr-12-0292

65. Hopkins MH, Owen J, Ahearn T, et al. Effects of supplemental vitamin D and calcium on biomarkers of inflammation in colorectal adenoma patients: a randomized, controlled clinical trial. Cancer Prev Res (Phila). 2011;4(10):1645-1654. doi:10.1158/1940-6207.CAPR-11-0105

66. Vaughan-Shaw PG, Timofeeva M, Ooi LY, et al. Differential genetic influences over colorectal cancer risk and gene expression in large bowel mucosa. Int J Cancer. 2021;149(5):1100-1108. doi:10.1002/ijc.33616

67. Chen L, Sun F, Yang X, et al. Correlation between RNA-Seq and microarrays results using TCGA data. Gene. 2017;628:200-204. doi:10.1016/j.gene.2017.07.056

68. Kogenaru S, Qing Y, Guo Y, et al. RNA-seq and microarray complement each other in transcriptome profiling. BMC Genom. 2012;13:629. doi:10.1186/1471-2164-13-629

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

How to cite this article: Vaughan-Shaw PG, Blackmur JP, Grimes G, et al. Vitamin D treatment induces in vitro and ex vivo transcriptomic changes indicating anti-tumor effects. FASEB J. 2022;36:e22082. doi:10.1096/fj.202101430RR