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The Wnt antagonist *DICKKOPF-1* gene is induced by 1α,25-dihydroxyvitamin D_3 associated to the differentiation of human colon cancer cells

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The Wnt–β-catenin pathway is aberrantly activated in most colon cancers. *DICKKOPF-1* (*DKK-1*) gene encodes an extracellular Wnt inhibitor that blocks the formation of signalling receptor complexes at the plasma membrane. We report that 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the most active vitamin D metabolite, increases the level of *DKK-1* RNA and protein in human SW480-ADH colon cancer cells. This effect is dose dependent, slow and depends on the presence of a transcription-competent nuclear vitamin D receptor (VDR). Accordingly, 1,25$(OH)_2D_3$ activates a 2300 bp fragment of the human *DKK-1* gene promoter. Chromatin immunoprecipitation assays revealed that 1,25$(OH)_2D_3$ treatment induced a pattern of histone modifications which is compatible with transcriptionally active chromatin. *DKK-1* is expressed at high level in colon cancer cell lines with a differentiated phenotype such as Caco-2 or HT-29. Exogenous expression of E-cadherin into SW480-ADH cells results in a strong adhesive phenotype and a 17-fold increase in *DKK-1* RNA. In contrast, an E-cadherin blocking antibody inhibits 1,25$(OH)_2D_3$-induced differentiation of SW480-ADH cells and *DKK-1* gene expression. Remarkably, *in vivo* treatment with the vitamin D analogue EB1089 induced DKK-1 protein expression in SW480-ADH cells xenografted in immunodeficient mice, and a correlation was observed in the expression of *VDR* and *DKK-1* RNA in a series of 32 human colorectal tumours. These data indicate that 1,25$(OH)_2D_3$ activates the transcription of the *DKK-1* gene, probably in an indirect way that is associated to the promotion of a differentiated phenotype. *DKK-1* gene induction constitutes a novel mechanism of inhibition of Wnt signalling and antitumour action by 1,25$(OH)_2D_3$.

Introduction

The family of Wnt glycoproteins regulates development and homeostasis activating several signalling pathways through binding to Frizzled receptors. The Wnt–β-catenin (canonical) pathway requires also the LDL receptor-related protein (LRP)5/6 co-receptors, which however are not involved in other β-catenin-independent (non-canonical) pathways (1–4). The Wnt–β-catenin-signalling pathway inhibits the phosphorylation of β-catenin by glycogen synthase kinase-3β and casein kinase I within a complex including the products of the tumour suppressor genes *adenomatous polyposis coli* and *AXIN*. This causes accumulation of β-catenin in the cytosol and its translocation to the nucleus, where β-catenin associates with DNA-bound T cell factor (TCF-1 to -4) regulating the transcription of proliferation and invasion genes (2,3). The Wnt–β-catenin pathway is aberrantly activated in most human colon cancers and a proportion of other carcinomas by mutation in *adenomatous polyposis coli*, or less frequently, *CTNNB1*/β-catenin or *AXIN1* (3,5). Non-canonical Wnt pathways involve the activation of the small GTPases Rho and Rac or kinases such as JNK, Ca^{2+}/calmodulin kinase II or protein kinase C or phospholipase C and phosphodiesterase via heterotrimeric GTP-binding proteins, but their relation to human cancer is unknown (6).

Several natural Wnt antagonists exist, including soluble Frizzled-related receptors, Wnt inhibitory factor and members of the DICK-KOPF (DKK-1 to -4) family (7). DKK-1 simultaneously binds to LRP5/6 and the transmembrane proteins Kremen 1/2 and induces LRP endocytosis, which prevents the formation of Wnt–Frizzled–LRP5/6 receptor complexes and blocks Wnt–β-catenin signalling (8–11). In the mouse small intestine and colon, forced *Dkk-1* expression inhibits the proliferation of the crypt progenitor cells that is induced by the transcriptional activity of β-catenin–TCF (12,13). Human *DKK-1* seems to have wide and complex effects on cell proliferation and differentiation: it induces the proliferation of human adult bone marrow stem cells (14) and inhibits osteoblastic differentiation (15), which is in line with the finding that high circulating levels of DKK-1 in patients with multiple myeloma are associated with osteolytic lesions (16). In addition, *DKK-1* expression is associated with adipocyte differentiation (17). Human *DKK-1* was reported as induced by p53 (18), although it has been shown to be induced by DNA damage and to sensitize to apoptosis in a p53-independent manner (19). *DKK-1* is also responsive to glucocorticoids in osteoblasts (20).

Interestingly, DKK-1 seems to have antitumoural effects independently of the antagonism of β-catenin–TCF transcriptional activity in H28 and MS-1 mesothelioma and HeLa cervical cancer cells (21,22). Also in DLD-1 colon cancer cells, which carry a truncated *adenomatous polyposis coli* gene and so have constitutively active the Wnt–β-catenin pathway, transfection of *DKK-1* decreases cell growth *in vitro* and tumour formation in immunodeficient mice (23). These data indicate that DKK-1 can inhibit tumourigenesis in different ways.

We and others have described that the transcription of the *DKK-1* gene is enhanced by β-catenin–TCF acting on several sites in the promoter region (24–26). Our group reported also that *DKK-1* is down-regulated in colon cancer (25) indicating the loss of a negative feedback control of the Wnt–β-catenin pathway in this neoplasia. More recently, this *DKK-1* down-regulation has been shown to be due at least in part to promoter methylation, which is specifically found in 25% of advanced, less differentiated tumours (Dukes' C and D) (23).

In this study, we report that the human *DKK-1* gene is induced by 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the most active metabolite of vitamin D, in colon cancer cells associated to the induction of an epithelial adhesive phenotype. Also *in vivo*, the 1,25$(OH)_2D_3$ analogue EB1089 increases DKK-1 expression in human xenografts. Moreover, we found a correlation between the expression of *DKK-1* and vitamin D receptor (*VDR*) RNA in a series of 32 human colorectal tumours. Our results indicate that the induction by 1,25$(OH)_2D_3$ is transcriptional but slow and probably indirect. *DKK-1* up-regulation is a novel action of 1,25$(OH)_2D_3$ that may contribute to inhibit Wnt–β-catenin-signalling pathway and to protect colon epithelial cells from malignant transformation.

Abbreviations: DKK-1, DICKKOPF; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; LRP, LDL receptor-related protein; PCR, polymerase chain reaction; TCF, T cell factor; VDR, vitamin D receptor.

Materials and methods

Cell lines

All cell lines used in this study were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 2 mM L-glutamine

(Invitrogen, Paisley, UK). SW480-ADH cells are a subpopulation derived from the SW480 cell line which show an adhesive phenotype, express substantial VDR levels and are thus responsive to $1,25(OH)_2D_3$ (27). All experiments using $1,25(OH)_2D_3$ (supplied by Dr Milan R.Uskokovic, BioXell, Nutley, NY and Drs R.Bouillon and M.Verstuyf, University of Leuven, Belgium and J.P.Van de Velde, Solvay-Duphar, Weesp, The Netherlands) were performed in Dulbecco's modified Eagle's medium supplemented with charcoal-treated serum. SW480-ADH cells were transfected with pcDNA3 and pBATEM2 at a molar ratio of 1:10 using Lipofectamine Plus (Invitrogen). The expression vector pBATEM2 encoding mouse full-length *CDH1*/E-cadherin cDNA under the control of the chicken β-actin promoter (28) was kindly provided by Dr M.Takeichi (Kyoto University, Kyoto, Japan). Stable transfectants were obtained after selection with 2 mg/ml G418 (Sigma, St Louis, MO) during 2 weeks. For disruption of E-cadherin-dependent cell–cell adhesion, the function-blocking antibody DECMA-1 (Sigma) was used at a concentration of 50 μg/ml.

Western blotting

Preparation of whole-cell extracts was as described elsewhere (27). Briefly, cells were lysed in RIPA buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 10% glycerol, 4 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 0.1% sodium dodecyl sulphate, 1% sodium deoxycholate and 50 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulphonic acid, pH 7.4 supplemented with a mixture of protease inhibitors: 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin). The protein concentration was measured using the Bio-Rad DC protein assay kit. For immunoblotting, cell lysates were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and the proteins were then transferred to BioTrace™ polyvinylidene difluoride membranes (Life Science, Pall Corporation). The membranes were incubated with the appropriate primary and secondary antibodies, and antibody binding was visualized using the enhanced chemiluminescence detection system (Amersham–GE Healthcare). We used mouse monoclonal antibodies against E-Cadherin (610182, BD Transduction Laboratories, Lexington, KY) and β-tubulin (T-4026, Sigma) and goat polyclonal antibodies against DKK-1 and β-actin (sc-14949 and sc-1616, respectively; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies used were HPR-conjugated anti-mouse IgG (H + L) (W402B, Promega, Madison, WI) and HPR-conjugated anti-goat IgG (sc-2020, Santa Cruz Biotechnology).

Northern blotting

Total RNA was prepared using the RNeasy kit (Qiagen, Hilden, Germany). Northern blots were performed according to the standard protocols. The *DKK-1* probe, a 0.5 kb HindIII fragment, was labelled by the random priming method (29). Hybridizations were carried out overnight at 42°C using ULTRAhyb® hybridization buffer (Ambion–Applied Biosystems, Warrington, UK). Methylene blue staining of 28S rRNA is shown as loading control.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were carried out as previously described (30). Chromatin was sheared to an average length of 0.2–2 kb. Polymerase chain reaction (PCR) amplification was performed in 25 μl with specific DKK-1 primers: 5′-TTTGTTGTCTCCCTCCCAAG-3′ (forward) and 5′-ATGACCGTCACTTTGCAAGC-3′ (reverse) that amplify a fragment of 195 bp between positions −119 and +76 of the transcription start site. Commercial histone H3 (acetyl K18) antibody (ab1191, Abcam, Cambridge, UK), histone H3 (trimethyl K4) antibody (ab8580, Abcam) and histone H3 (trimethyl K9) antibody (ab8898, Abcam) were used. GAPDH was analysed as an internal control using specific primers: 5′-TCTTCTTTTGCGCCAG-3′ (forward) and 5′-AGCCCCAGCCTTCTCCA-3′ (reverse). The sensitivity of PCR amplification was evaluated on serial dilutions of total DNA collected after sonication (input fraction).

Immunofluorescence and immunohistochemistry

Immunofluorescence studies were done as described (27) using antibodies against DKK-1 (1:1000; sc-14949, Santa Cruz Biotechnology). For immunohistochemistry, we used severe immune-deficient female *scid* mice obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were subcutaneously injected with 5×10^6 Snail1 (SNAIL1-HA) or Mock-infected (Mock) SW480-ADH cells in each flank and treated with EB1089 or placebo as described previously (31). The maintenance and handling of animals were as recommended by the European Union (ECC Directive of 24 November 1986, 86/609/EEC) and all experiments were approved by the Animal Experimentation Committee at our Institute. Every effort was made to minimize animal suffering and to reduce the number of animals used. Immunostaining of formalin-fixed, paraffin-embedded tumour sections was performed as described (32) using appropriate dilutions of the anti-DKK-1 antibody (sc-14949, Santa Cruz Biotechnology). Signal specificity was ensured by parallel pre-incubation of the antibody with blocking peptide (sc-14949P, Santa Cruz Biotechnology) for 30 min at 4°C. Images were captured with an Olympus DP70 digital camera mounted on a Zeiss Axiophot microscope equipped with epifluorescence (immunofluorescence images) or with a Canon Power Shot G5 digital camera mounted on a Zeiss Axioskop2 plus microscope (immunohistochemistry images). All images were processed using Adobe Photoshop software. Quantification of signals in immunohistochemistry analyses was performed using Soft Imaging System software (Olympus Soft Imaging Solutions, Münster, Germany) according to the manufacturer's instructions.

Reporter assays

The pGL3basic-DKK-1 promoter construct has been described previously (25). In all, 5×10^4 cells per well on 24-well tissue culture plates were seeded 24 h before transfection. All cell lines were transfected using the jetPEI reagent (PolyPlus Transfection, Illkirch, France) and harvested 48 h later for analysis of luciferase activities. *Firefly* and *Renilla* luciferase activities were separately measured using the Dual Luciferase reagent kit (Promega) and a Lumat LB9507 luminometer (Berthold). Results shown are mean + standard deviations of six replicates.

Patients, samples and RNA extraction

We recruited 32 patients diagnosed as bearing colorectal cancer and were included in a study approved by the Research Ethics Board of our hospital. Patients were considered sporadic cases because no clinical antecedents of familial adenomatous polyposis were reported and those with clinical criteria of hereditary non-polyposis colorectal cancer (Amsterdam criteria) were excluded. Normal and tumour tissue samples were obtained immediately after surgery, immersed in RNA*later*™ (Ambion–Applied Biosystems), snap-frozen in liquid nitrogen and stored at −80°C until processing. All patients of the study gave written informed consent. Tissue RNA was extracted from ∼30 mg of tumour or normal samples using RNeasy Mini kit (Qiagen).

Real-time PCR

Logarithms of the ratios of *DKK-1*, *CDH1*/E-cadherin and *VDR* RNA levels in tumour and normal tissue or in cells treated with $1,25(OH)_2D_3$ or vehicle were estimated by quantitative real-time PCR using the following primers: *DKK1*, 5′-GATCATAGCACCTTGGATGGG-3′ (forward) and 5′-GGCACAGTCTGATGACCGG-3′ (reverse); *CDH1*/E-cadherin, 5′-AGAACGCATTGCCACATACACTC-3′ (forward) and 5′-CATTCTGATCGGTTACCGTGATC-3′ (reverse) and *VDR*, 5′-TTGCCATACTGCTGGACGC-3′ (forward) and 5′-GGCTCCCTCCACCATCATT-3′ (reverse). Values were calculated in samples in a relative quantification where the amount of the targets was expressed in relation to the geometric average of two reference housekeeping genes: *succinate dehydrogenase complex subunit A* and *ubiquitin C* as described (33). The relative concentrations of target and reference genes were calculated by interpolation using a standard curve generated with a serial dilution of a cDNA prepared from RNA extracted from MCF-7 cells. For the synthesis of the first strand of cDNA, 400 ng of total RNA was retrotranscribed using the Gold RNA PCR Core kit (Applied Biosystems) following the manufacturer's instructions. Random hexamers were used as primers for cDNA synthesis.

Real-time PCR was performed in a LightCycler apparatus (Roche Diagnostics, Mannheim, Germany) using the LightCycler-FastStart^{PLUS} DNA Master SYBR Green I kit (Roche Diagnostics). Each reaction was performed in a final volume of 20 μl containing 2 μl of the cDNA product sample, 0.5 μM of each primer and 1× reaction mix including FastStar DNA polymerase, reaction buffer, deoxycytidine triphosphates and SYBR green. Thermal cycling for all genes was initiated with a denaturing step at 95°C for 10 min and followed by 40 cycles (denaturing at 94°C for 0 s, annealing at 59°C for 5 s and elongation at 72°C for 5 s, in which fluorescence was acquired). At the end of the PCR cycles, melting curve analyses were performed as well as electrophoresis of the products on non-denaturing 8% polyacrylamide gels, followed by sequencing, in order to validate the generation of the specific PCR product expected.

Data analysis

The tumour to normal ratios of gene expression were not normally distributed (Kolmogorov–Smirnov test, Lilliefors correction). For this reason, we normalized the data distribution by using $\log_{10}$ for statistical analysis. For the same reason, we also used the geometric (rather than the arithmetic) average of the tumour and normal to describe the expression gene data. The correlations between gene expression levels were studied using the Spearman correlation coefficient (ρ).

Results

We used quantitative RT–PCR to validate a recent transcriptome analysis of human SW480-ADH colon cancer cells that revealed a 2.6-fold increase in *DKK-1* RNA level following a 2-day treatment

with $1,25(OH)_2D_3$. Time-course and dose-curve experiments showed that $1,25(OH)_2D_3$ (10^{-7} M) caused a slow 3- to 5-fold induction of *DKK-1* RNA at 24–48 h upon treatment (Figure 1A and B). The effect of $1,25(OH)_2D_3$ was specific, as several hormones (dexamethasone, retinoic acid, progesterone and oestradiol) acting through members of the superfamily of nuclear receptors similar to VDR did not induce *DKK-1* (Figure 1C). The induction of *DKK-1* was confirmed at the protein level (Figure 1D) and in another colon cancer cell line (LS-174T; data not shown). Immunofluorescence studies confirmed the increase in DKK-1 protein expression following $1,25(OH)_2D_3$ exposure and showed its preferential localization in the cell periphery, Golgi apparatus and vesicles of the exocytic route (Figure 1E). These results confirmed that $1,25(OH)_2D_3$ induces *DKK-1* expression with slow kinetics, which precluded the use of translation inhibitors such as cycloheximide to investigate whether the induction is direct or indirect.

To examine whether VDR mediates the modulation of *DKK-1* expression by $1,25(OH)_2D_3$, we first checked that very little induction takes place in SW480-R cells expressing hardly detectable VDR (27) (Figure 2A). Furthermore, no induction was evident in SW480-ADH cells stably expressing the Snail1 transcription factor (SNAIL1-HA), which is a potent repressor of *VDR* expression in this cell line (31) (Figure 2B). Next, we investigated whether $1,25(OH)_2D_3$ changed *DKK-1* transcription. A statistically significant activation (up to 30%, $P < 0.001$) of a 2.3 kb fragment of the human *DKK-1* promoter (25) was found in SW480-ADH cells (Figure 3A). In accordance also with a transcriptional activation, the cotransfection of VDR-negative HEK 293T cells with a wild-type VDR but not with a mutant version (ΔAF2-VDR) lacking the C-terminal AF-2 region responsible for transcriptional activation allowed induction of the *DKK-1* promoter by $1,25(OH)_2D_3$ (Figure 3B). Finally, chromatin immunoprecipitation assays showed that exposure to $1,25(OH)_2D_3$ caused an increase in the level of histone 3 acetylation and trimethylation at Lys 4 (TriMetK4H3), which are linked to activation of transcription (Figure 3C). Accordingly, we did not detect histone 3 trimethylation at Lys 9 (TriMetK9H3) which is linked to transcriptional silencing. In addition, no VDR binding to the *DKK-1* promoter region studied was detected. In contrast, $1,25(OH)_2D_3$ did not change the stability of *DKK-1* mRNA ($t_{1/2}$ = 2 h) in SW480-ADH cells, as assessed by time-course real-time RT–PCR analysis (data not shown). Collectively, our results indicate that $1,25(OH)_2D_3$ promotes epigenetic chromatin changes upstream the *DKK-1* coding region that are compatible with the transcriptional activation of the gene. As *DKK-1*

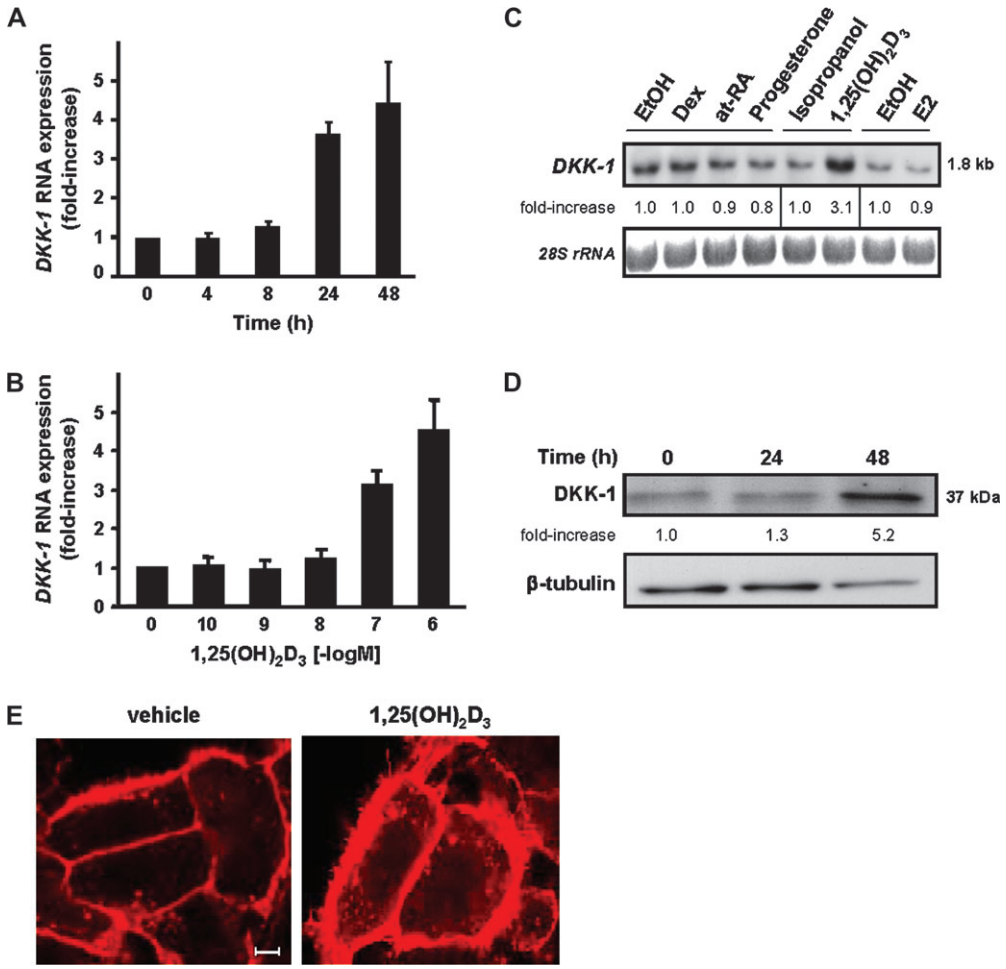

Fig. 1. $1,25(OH)_2D_3$ induces *DKK-1* gene expression. (**A**) Kinetics of *DKK-1* RNA induction in SW480-ADH cells treated with $1,25(OH)_2D_3$ (10^{-7} M) for the indicated times. *DKK-1* RNA expression was analysed using quantitative RT–PCR. Normalized mean values and standard deviation from three independent experiments are shown. (**B**) Dose-curve effect of $1,25(OH)_2D_3$ on *DKK-1* RNA expression. Cells were treated with the indicated concentrations of $1,25(OH)_2D_3$ and *DKK-1* induction analysed as in (A). (**C**) Specificity of $1,25(OH)_2D_3$ action. Northern blot analysis of *DKK-1* mRNA expression in SW480-ADH cells treated with 10^{-7} M dexamethasone (Dex), all-trans retinoic acid (at-RA), progesterone, $1,25(OH)_2D_3$ or oestradiol (E2) for 48 h. Controls using the corresponding vehicles (ethanol, EtOH or isopropanol) in normal or phenol red-free medium (E2) are shown. 28S rRNA was used as loading control. (**D**) DKK-1 protein is also induced by $1,25(OH)_2D_3$. SW480-ADH cells were treated with $1,25(OH)_2D_3$ (10^{-7} M) for the indicated times and extracts analysed by western blot. β-Tubulin was used as loading control. (**E**) Immunofluorescence analysis of DKK-1 protein expression in SW480-ADH cells treated with $1,25(OH)_2D_3$ (10^{-7} M) or vehicle for 48 h. Bar, 2.5 μm.

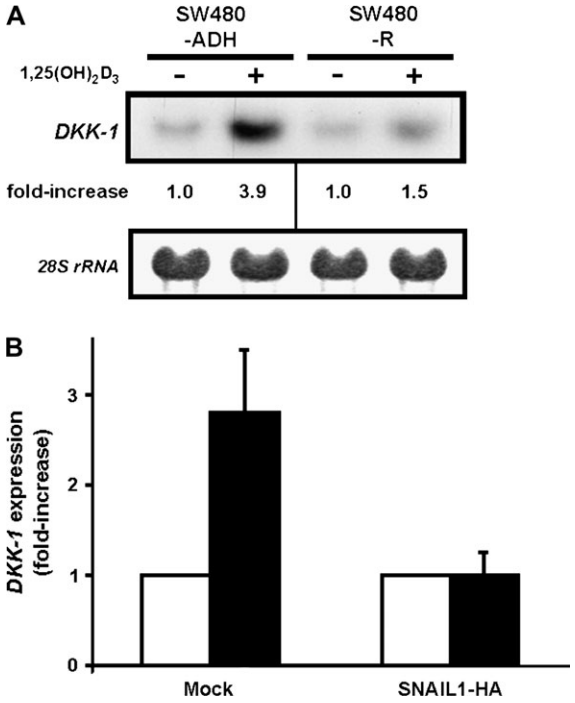

Fig. 2. VDR mediates the induction of *DKK-1* by $1,25(OH)_2D_3$. (**A**) $1,25(OH)_2D_3$ induces *DKK-1* RNA expression in VDR-positive SW480-ADH cells but not in VDR-negative SW480-R cells. Northern blot analysis of cells treated with $1,25(OH)_2D_3$ (10^{-7} M) or vehicle for 48 h. 28S rRNA was used as loading control. (**B**) Snail1 blocks induction of *DKK-1* RNA by $1,25(OH)_2D_3$. SW480-ADH cells stably expressing Snail1 (SNAIL1-HA) or a mock construct (Mock) were treated with $1,25(OH)_2D_3$ (10^{-7} M, black columns) or vehicle (white columns) for 48 h. *DKK-1* RNA expression was analysed using quantitative RT–PCR. Mean values and standard deviation from three independent experiments are shown as fold increase relative to untreated cells.

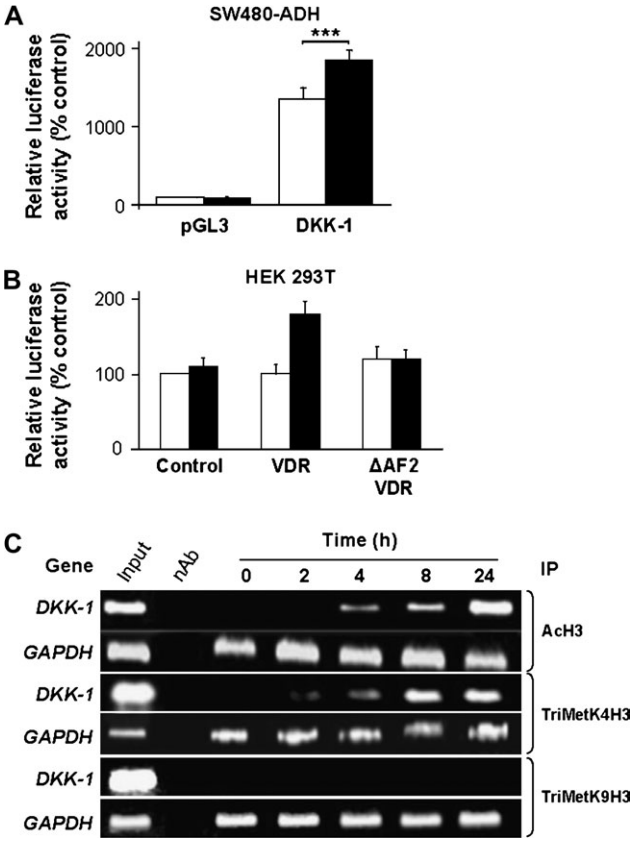

Fig. 3. $1,25(OH)_2D_3$ induces *DKK-1* gene transcription. (**A**) Activation of the human *DKK-1* gene promoter (−2236/+112) by $1,25(OH)_2D_3$. SW480-ADH cells were transfected with either the *DKK-1* promoter (100 ng per well) or the control pGL3-basic plasmid (100 ng per well). Transfections were performed as described in Materials and methods. Cells were treated with vehicle (white colums) or $1,25(OH)_2D_3$ (10^{-7} M, black columns) during 48 h after transfection. Mean values and standard deviation corresponding to three independent experiments performed in hexaplicate are shown, $^{***}P < 0.001$. (**B**) Activation of the *DKK-1* promoter requires a transcriptionally competent VDR. HEK 293T cells were transfected with the *DKK-1* promoter (50 ng per well) together with plasmids encoding VDR (50 ng per well), ΔAF2-VDR (lacking the C-terminus transactivation domain, 50 ng per well) or an empty vector (control, 50 ng per well) and treated with 10^{-7} M $1,25(OH)_2D_3$ (black columns) or vehicle (white columns) as in (A). Mean values and standard deviation corresponding to three independent experiments performed in hexaplicate are shown. (**C**) $1,25(OH)_2D_3$ induces a transcriptionally active *DKK-1* chromatin structure. Chromatin immunoprecipitation analysis of the histone modification status of the *DKK-1* promoter at the indicated times after $1,25(OH)_2D_3$ (10^{-7} M) treatment of SW480-ADH cells. The input and bound fractions are shown. No antibody (nAb) samples are shown as negative control and *GAPDH* as an internal control. AcH3, acetyl histone 3; TriMetK4H3, tri-methyl lysine 4 histone 3 and TriMetK9H3, tri-methyl lysine 9 histone 3.

induction by $1,25(OH)_2D_3$ requires VDR but no binding of VDR to the region of the *DKK-1* promoter studied was detected, the effect of $1,25(OH)_2D_3$ on *DKK-1* transcription is probably indirect.

To explore how general the regulation of *DKK-1* expression by $1,25(OH)_2D_3$ was, we screened by RT–PCR a panel of 12 human colon cancer cell lines. Basal *DKK-1* RNA levels were high in those lines with a differentiated adhesive epithelial phenotype (HT-29, Caco-2, HCT116) and low or absent in those with less differentiated (SW480-ADH, LS-174T, LoVo, SW1417, DLD-1) or highly undifferentiated (SW620, SW48, COLO 205, RKO) phenotype. These results were in overall agreement with the respective methylation status of the *DKK-1* gene promoter (23) and also in relation with the stage of the tumours from which they were established (Table I). Not unexpectedly, $1,25(OH)_2D_3$ neither increased the expression of *DKK-1* in those cells which already showed high levels nor induced *DKK-1* RNA in cell lines in which the gene promoter is methylated or express low levels of VDR (Table I). To confirm that *DKK-1* expression is linked to the differentiated phenotype of colon cancer cells, we analysed the effect of the revertion to an epithelial adhesive phenotype of SW480-ADH cells by re-introduction of E-cadherin. SW480-ADH cells stably transfected with an exogenous *CDH1*/E-cadherin gene (SW480-ADH–E-cadherin) showed a strong adhesive phenoptype (Figure 4A), very high E-cadherin expression (Figure 4B), and much higher (17-fold) basal *DKK-1* RNA level than control vector-transfected cells (Figure 4C). Interestingly, as it happens in high-expressing colon cancer cell lines, no further increase in *DKK-1* expression was found following exposure of SW480-ADH–E-cadherin cells to $1,25(OH)_2D_3$ (Figure 4C). We have previously shown that $1,25(OH)_2D_3$-induced differentiation of SW480-ADH cells is linked to the induction of E-cadherin and its localization at plasma membrane adherent junctions (27). Treatment of these cells with a blocking antibody against E-cadherin (DECMA-1) results in partial inhibition of $1,25(OH)_2D_3$-induced differentiation (Figure 4D) and in a substantial decrease in *DKK-1* gene induction (Figure 4E).

Next, we studied the regulation of *DKK-1 in vivo*. In agreement with the results in cultured cells, the less hypercalcemic $1,25(OH)_2D_3$ analogue EB1089 induced DKK-1 protein expression in xenografts generated by SW480-ADH cells (Mock; Figure 5A). Emphasizing the VDR-mediated transcriptional effect of EB1089, no changes in DKK-1 expression were found in tumours generated by cells expressing Snail1 (SNAIL1-HA) after treatment with the analogue (Figure 5A). However, these xenografts showed an overall reduced expression of DKK-1 protein that may be linked to the less differentiated phenotype of SNAIL1-HA cells when compared with the parental cell line (31).

Table I. Expression of *DKK-1* in a series of colon cancer cell lines

Cell line	Basal expression			$1,25(OH)_2D_3$ effect	*DKK-1* methylation[b]	Dukes' stage[c]	Differentiation
	DKK-1 RNA	VDR[a] protein	E-cadherin protein				
HT-29	+++	+	+++	−	No	Unknown	Well
Caco-2	+++	+	++	−	No	Unknown	Well
HCT116	++	+	++	−	No	Unknown	Well
LS-174T	+	++	−[d]	+	No	B	Medium
SW480-ADH	+	++	−/+[e]	++	No	B	Medium
SW620	+	−	−/+	−	No	C	Poor
LoVo	+	+	++	−	No	C	Medium
SW1417	+	++	+++	−	No	C	Medium
DLD-1	−	++	+++	−	Yes	C	Medium
SW48	−	−	++[f]	−	Yes	C	Poor
COLO 205	−	−	++[g]	−	Yes	D	Poor
RKO	−	−	−	−	Yes	Unknown	Poor

[a]VDR expression is only indicative of the cellular response to $1,25(OH)_2D_3$, which depends also on several other factors [i.e. levels of co-activators and co-repressors, CYP24 and CYP27B1 activity (39)].
[b]See ref. (23).
[c]According to the American Type Culture Collection.
[d]*CDH1*/E-cadherin RNA is expressed but mutated and is not translated into protein (45).
[e]E-cadherin expression is highly stimulated in SW480-ADH cell line by $1,25(OH)_2D_3$ (28).
[f]SW48 cells express E-cadherin but, however, it does not localize at the plasma membrane due to mutation of p120-catenin (46).
[g]COLO 205 cells expose functional E-cadherin molecules on their surface but fail to organize compact cell aggregates (47).

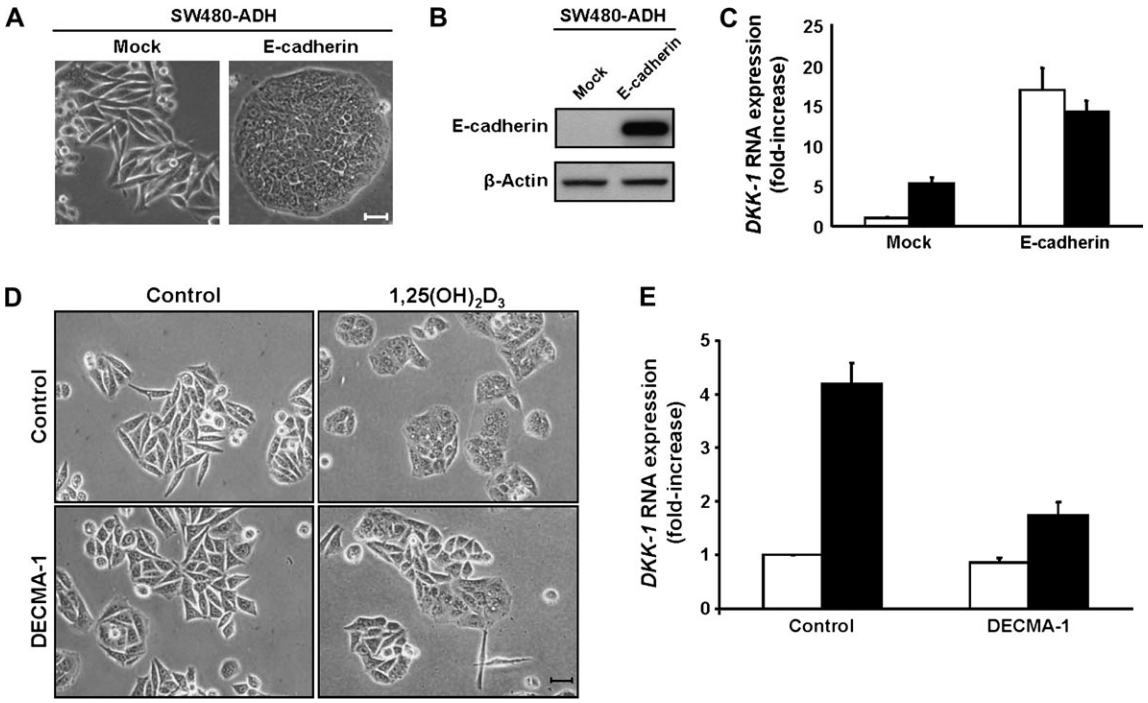

Fig. 4. *DKK-1* expression is induced by E-cadherin-mediated differentiation. (**A**) SW480-ADH cells stably expressing E-cadherin display a well-differentiated phenotype. Phase-contrast micrographs of mock (left) and E-cadherin-transfected (right) SW480-ADH cells. Bar, 20 μm. (**B**) Western blot analysis of E-cadherin expression in stably transfected SW480-ADH cells. β-Actin was used as loading control. (**C**) E-cadherin increases basal and $1,25(OH)_2D_3$-induced *DKK-1* RNA levels. SW480-ADH mock or E-cadherin-expressing cells were treated with 10^{-7} M $1,25(OH)_2D_3$ (black columns) or vehicle (white columns) for 48 h. Total RNA was purified and *DKK-1* mRNA quantified by quantitative RT–PCR. Mean values and standard deviation from three independent experiments normalized as described previously are shown. (**D**) Phase-contrast micrographs of SW480-ADH cells treated (right panels) or not (left panels) with 10^{-7} M $1,25(OH)_2D_3$ for 24 h in the presence of the E-cadherin blocking antibody (DECMA-1, 50 μg/ml; bottom panels) or a control antibody (top panels). Bar, 20 μm. (**E**) DECMA-1 antibody inhibits the induction of *DKK-1* mRNA by $1,25(OH)_2D_3$. Total RNA from cells treated as in (D) was purified and *DKK-1* mRNA quantified by real-time RT–PCR. Mean values and standard deviation from three independent experiments normalized as described previously are shown.

Finally, the analysis by real-time RT–PCR of biopsies of normal and tumoural tissue from a series of 32 patients revealed a statistically significant correlation between the expression of *VDR* and *DKK-1* in human colon cancer ($\rho = 0.445$, $P = 0.011$) (Figure 5B, left). A tendency to a direct relation that did not reach statistical significance was found between the RNA expression levels of *DKK-1* and *CDH1*/E-cadherin ($\rho = 0.238$, $P = 0.190$) (Figure 5B, right).

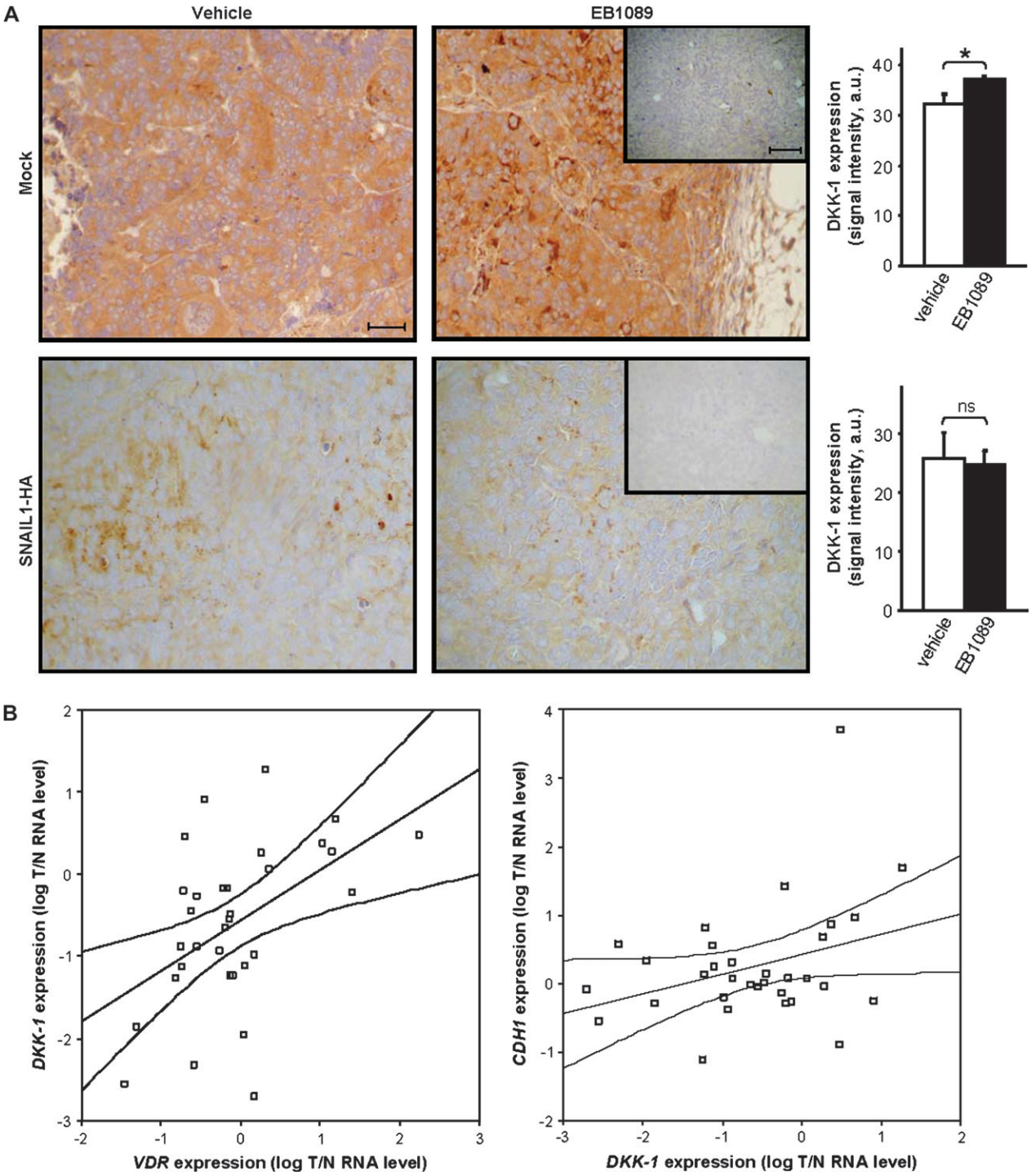

Fig. 5. *In vivo* studies of the regulation of human DKK-1 by $1,25(OH)_2D_3$. (**A**) EB1089 induces DKK-1 in human xenografts. Representative sections of tumours grown in mice injected with mock-infected (Mock) or Snail1-expressing (SNAIL1-HA) SW480-ADH cells that were treated with EB1089 (10^{-7} M) or vehicle as described in Materials and methods. Sections were immunostained with antibodies against DKK-1. Insets correspond to sections stained with antibody pre-incubated with DKK-1 blocking peptide. Scale bar, 25 μm; inset, 250 μm. Quantification of DKK-1 signal is shown (right). Mean values and standard deviation of signals from three fields are shown. $^*P < 0.05$; ns, non-significant. (**B**) Analysis of the relation between the expression of *DKK-1* and *VDR* RNA (left) and that of *DKK-1* and *CDH1*/E-cadherin RNA (right) in human colon tumours. RNA levels in normal (N) and tumour (T) samples from 32 patients were estimated by real-time RT–PCR and normalized as described in Materials and methods. The relation between the $\log_{10}$ tumour to normal ratio of each patient is shown: for *DKK-1* and *VDR*, Spearman $\rho = 0.445$; $P = 0.011$ and for *DKK-1* and *CDH1*/E-cadherin, Spearman $\rho = 0.238$; $P = 0.190$.

Discussion

The Wnt–β-catenin signalling pathway plays crucial roles in development and its aberrant activation is an initial and crucial event in the majority of colon cancers (3,5,34). Recent studies showed the silencing due to promoter methylation of genes encoding endogenous inhibitors of this pathway (*soluble Frizzled-related receptor proteins* and *DKK-1*) in a proportion of human colorectal tumours, which may contribute to its deregulation (23,35,36). Here, we present evidence

that the pleiotropic hormone $1,25(OH)_2D_3$ induces the expression of *DKK-1* RNA and protein in human colon cancer cells and in xenografts. Furthermore, we show a correlation between *VDR* and *DKK-1* RNA levels in human colorectal tumours that suggests the regulation also in patients.

$1,25(OH)_2D_3$ has wide antitumour effects that include the induction of differentiation, the inhibition of cell proliferation and invasion, the sensitization to pro-apoptotic stimuli and the inhibition of angiogenesis and metastasis in many types of cancer (37,38, reviews). Indeed, several antitumour effects of $1,25(OH)_2D_3$ such as the activation of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ cell cycle inhibitors and the modulation of the apoptosis related genes *BAK*, *BAX* and *BAG1* have been described in cultured cancer cells, and the antitumour activity has been also extensively confirmed in spontaneous and chemically induced experimental carcinogenesis in mice (38,39, reviews). Globally, the wide effects of $1,25(OH)_2D_3$ on the gene expression profile of SW480-ADH cells is in agreement with a restoration of the normal differentiated phenotype (40).

Work by our group revealed that $1,25(OH)_2D_3$ antagonizes the Wnt–β-catenin-signalling pathway by (i) promoting the binding of VDR to β-catenin, thus preventing the formation of transcriptionally active β-catenin–TCF complexes and (ii) the induction of *CDH1*/E-cadherin transcription, which favours the nuclear export of β-catenin and its relocalization bound to E-cadherin at the plasma membrane adherens junctions (27). Shah *et al.* (41) have recently characterized the molecular basis of the VDR–β-catenin interaction. Our finding that $1,25(OH)_2D_3$ induces *DKK-1* expression represents a third mechanism by which this hormone antagonizes the Wnt–β-catenin pathway. Moreover, as several recent reports indicate that DKK-1 has antitumoural effects independently of β-catenin (21–23), the novel action described here may be of unforeseen importance for the anticancer action of $1,25(OH)_2D_3$. The existence of several mechanisms of Wnt–β-catenin signalling antagonism by $1,25(OH)_2D_3$ reveals the importance of this pathway and of its regulation for the biology of the colonic epithelium.

The regulation of *DKK-1* expression by $1,25(OH)_2D_3$ appears to be transcriptional but indirect. The slow kinetics of *DKK-1* RNA accumulation and the lack of VDR binding to the promoter region that is activated by the hormone together with the lack of effect on the half-life of *DKK-1* RNA and the requirement of VDR transcriptional activity strongly suggest that $1,25(OH)_2D_3$ up-regulates the transcription of *DKK-1* via intermediate proteins encoded by early responsive genes that remain uncharacterized. Although we cannot rule out that VDR might regulate directly the *DKK-1* gene promoter acting with slow kinetics on regulatory regions located far from the transcription initiation site, the finding that *DKK-1* is up-regulated by ectopic E-cadherin in SW480-ADH cells and that a blocking antibody against E-cadherin inhibits $1,25(OH)_2D_3$-mediated *DKK-1* induction suggests that the regulatory effect of $1,25(OH)_2D_3$ is an indirect consequence of the induction of the epithelial adhesive phenotype. We have previously reported that *DKK-1* expression is silenced by promoter methylation in nearly 25% colorectal tumours in advanced stages of progression (Dukes' C and D), which are typically dedifferentiated (23). Since our results suggest an association of *DKK-1* with the differentiated phenotype, one interesting hypothesis is that *DKK-1* silencing is not only concomitant but also plays a role in the dedifferentiation process. Interestingly, VDR expression has been reported to be a marker of differentiation in colon carcinoma cells (42,43) and is lost through colon cancer progression together with that of E-cadherin in parallel to the up-regulation of *SNAIL1* (31,43,44). This may so explain the correlation between *DKK-1* and *VDR* expression in human tumours. The basis for the increased *DKK-1* expression in cells over-expressing E-cadherin is unknown. It is conceivable that strong intercellular adhesion caused by high E-cadherin levels may signal to the nucleus being responsible for *DKK-1* induction but no data are presently available. However, E-cadherin/differentiation-independent effects of $1,25(OH)_2D_3$ must also cooperate, as *DKK-1* gene promoter is also induced in HEK 293T cells that lack E-cadherin and do not differentiate in response to $1,25(OH)_2D_3$ treatment.

In conclusion, our results show $1,25(OH)_2D_3$ as a novel regulator of *DKK-1* expression, and thus of Wnt–β-catenin-signalling pathway in colon epithelial cells. The finding that DKK-1 exerts antitumour actions other than the inhibition of signalling from the Wnt-receptor complex, for instance on cells harbouring intracellular mutations in the pathway, or even unrelated to the transcriptional activity of β-catenin–TCF (21–23) focus a great interest on the study of DKK-1 as a potential mediator of at least part of the complex anticancer activity of $1,25(OH)_2D_3$.

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