The inhibition of Wnt/ β -catenin signalling by $1\alpha,25$ -dihydroxyvitamin D_3 is abrogated by Snail1 in human colon cancer cells

María Jesús Larriba, Noelia Valle, Héctor G Pálmer, Paloma Ordóñez-Morán, Silvia Álvarez-Díaz, Karl-Friedrich Becker¹, Carlos Gamallo², Antonio García de Herreros³, José Manuel González-Sancho and Alberto Muñoz

Instituto de Investigaciones Biomédicas 'Alberto Sols', Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Arturo Duperier 4, E-28029 Madrid, Spain ¹Institut für Pathologie, Technische Universität München, Trogerstrasse 18, D-81675 München, Germany

²Hospital Universitario de la Princesa, Diego de León 62, E-28006 Madrid, Spain

³Institut Municipal d'Investigació Mèdica-Universitat Pompeu-Fabra, Doctor Aiguader 80, E-08003 Barcelona, Spain

(Requests for offprints should be addressed to A Muñoz; Email: amunoz@iib.uam.es)

M J Larriba, N Valle and H G Pálmer contributed equally to this work.

H G Pálmer is now at Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

Abstract

The Wnt/ β -catenin signalling pathway is activated in 90% of human colon cancers by nuclear accumulation of β -catenin protein due to its own mutation or to that of adenomatous polyposis coli. In the nucleus, β -catenin regulates gene expression promoting cell proliferation, migration and invasiveness. $1\alpha,25$ -dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) inhibits β -catenin signalling by inducing its binding to vitamin D receptor (VDR) and by promoting β -catenin nuclear export. The transcription factor Snail1 represses VDR expression and we demonstrate here that Snail1 also abolishes the nuclear export of β -catenin induced by $1,25(OH)_2D_3$ in SW480-ADH cells. Accordingly, Snail1 relieves the inhibition exerted by $1,25(OH)_2D_3$ on genes whose expression is driven by β -catenin, such as *c-MYC*, ectodermal-neural cortex-1 (*ENC-1*) or ephrin receptor B2 (*EPHB2*). In addition, Snail1 abrogates the inhibitory effect of $1,25(OH)_2D_3$ on cell proliferation and migration. In xenografted mice, Snail1 impedes the nuclear export of β -catenin and the inhibition of *ENC-1* expression induced by EB1089, a $1,25(OH)_2D_3$ analogue. The elevation of endogenous SNAIL1 protein levels reproduces the effect of an ectopic *Snail1* gene. Remarkably, the expression of exogenous VDR in cells with high levels of Snail1 normalizes the transcriptional responses to $1,25(OH)_2D_3$. However, this exogenous VDR failed to fully restore the blockage of the Wnt/ β -catenin pathway by $1,25(OH)_2D_3$. This suggests that the effects of Snail1 on this pathway are not merely due to the repression of *VDR* gene. We conclude that Snail1 is a positive regulator of the Wnt/ β -catenin signalling pathway in part through the abrogation of the inhibitory action of $1,25(OH)_2D_3$.

Endocrine-Related Cancer (2007) 14 141–151

Introduction

There are epidemiological data indicating that vitamin D intake and exposure to sunlight exerts a protective effect against colorectal carcinogenesis and other neoplasias (Grant & Garland 2004, review). $1\alpha,25$ -dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the most active vitamin D metabolite, is a hormone that is synthesised in the kidney and by several other cell types that exerts pleiotropic effects in the organism. In addition to its classical role regulating calcium and phosphate

homeostasis and bone biology, $1,25(OH)_2D_3$ has anti-proliferative, pro-apoptotic and pro-differentiation effects on many tumour cells (Hansen *et al.* 2001, Ordóñez-Morán *et al.* 2005, reviews). Furthermore, $1,25(OH)_2D_3$ displays anti-tumoral action in experimental animals also based on its anti-invasive, anti-angiogenic and anti-metastatic activity (Ordóñez-Morán *et al.* 2005, review). While the clinical use of $1,25(OH)_2D_3$ is hampered by its hypercalcemic effect, a number of analogues with reduced calcemic properties

are being used in pre-clinical studies and clinical trials against colon cancer and other neoplasias.

Colon cancer is one of the most frequent neoplasias and a major health problem worldwide. Wnt/ β -catenin signalling pathway is activated in 90% of human colon cancers. Indeed, nearly all sporadic colorectal tumours and also those developed by patients with familial adenomatous polyposis contain mutations in the adenomatous polyposis coli (APC) tumour suppressor gene or, less frequently, in the *CTNNB1* gene encoding β -catenin (Sancho et al. 2004, review). The β -catenin protein is a component of intercellular adherens junctions where it binds to E-cadherin (Gumbiner 1996, Jamora & Fuchs 2002, reviews). However, when it is released from these junctions, β -catenin is rapidly targeted for degradation by a complex involving APC, axin and the protein kinases glycogen synthase kinase (GSK)-3 β and casein kinase I. Mutations in either *APC* or *CTNNB1*, or stimulation with Wnt ligands leads to β -catenin accumulation in the cytoplasm and its translocation to the cell nucleus. In the nucleus, β -catenin binds to proteins of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family (TCF4 in colon epithelial cells) and it regulates the expression of genes involved in proliferation, invasiveness and angiogenesis. In this way, β -catenin is responsible for imposing an undifferentiated progenitor phenotype on cells, thereby promoting the progression of colon cancers (van de Wetering et al. 2002, Giles et al. 2003, Sancho et al. 2004).

Most, if not all, 1,25(OH) $_2$ D $_3$ actions are mediated through its binding to the vitamin D receptor (VDR), a ligand-modulated transcription factor that belongs to the nuclear hormone receptor superfamily. We have seen that 1,25(OH) $_2$ D $_3$ and several non-hypercalcemic analogues promote differentiation and inhibit the proliferation of human colon cancer cells in culture. These effects are linked to the induction of E-cadherin expression and the blockage of β -catenin transcriptional activity, counteracting the expression of β -catenin target genes (Pálmer et al. 2001). These effects of 1,25(OH) $_2$ D $_3$ are based on the rapid induction of the VDR/ β -catenin interaction and the subsequent expression of E-cadherin that promotes the redistribution of β -catenin to the plasma membrane (Pálmer et al. 2001). We have seen that the Snail1 transcription factor binds to, and represses the human *VDR* gene promoter and that elevated *SNAIL1* expression correlates with low *VDR* expression in human colon cancers (Pálmer et al. 2004, Peña et al. 2005). Snail1 is a zinc-finger transcription factor involved in processes that facilitate cell movement during embryonic development, as well as, in pathological processes such as tumour invasion (Barrallo-Gimeno & Nieto 2005,

review). Snail1 expression leads to the acquisition of fibroblastic properties by epithelial cells (epithelial-to-mesenchymal transition, EMT) facilitating their migration (Batlle et al. 2000, Cano et al. 2000). Snail1 represses the expression of the invasion suppressor *CDH1*/E-cadherin gene (Batlle et al. 2000, Cano et al. 2000), a crucial event for EMT. Up-regulation of the *SNAIL1* gene has been observed in gastric tumours and melanomas (Poser et al. 2001, Rosivatz et al. 2002), breast, hepatocellular and colon carcinomas (Blanco et al. 2002, Jiao et al. 2002, Pálmer et al. 2004, Peña et al. 2005, Roy et al. 2005), and in synovial sarcomas (Saito et al. 2004); and it is frequently linked to the acquisition of undifferentiated and invasive phenotypes. Furthermore, Snail1 has recently been proposed to be responsible for local recurrence of breast cancer (Moody et al. 2005).

In this study, we show that Snail1 abrogates the inhibitory activity of 1,25(OH) $_2$ D $_3$ on β -catenin nuclear signalling and on the gene expression driven by this pathway. Likewise, Snail1 reverses the effects of 1,25(OH) $_2$ D $_3$ on the proliferation and migration of colon cancer cells. Furthermore, Snail1 promotes the nuclear localization of β -catenin in human xenografts grown in immune-deficient mice that have been treated with EB1089, a 1,25(OH) $_2$ D $_3$ analogue. Additionally, the expression of Snail1 prevents EB1089 from inhibiting the expression of β -catenin/TCF target genes such as *ENC-1* (ectodermal-neural cortex-1). Interestingly, ectopic expression of VDR in cells with elevated Snail1 expression fully restores their responsiveness to the gene regulatory activity of 1,25(OH) $_2$ D $_3$ but not to the inhibition of the Wnt/ β -catenin signalling pathway.

Materials and methods

Gene transfer and cell culture

SW480-ADH cells retrovirally transduced with the mouse *Snail1* cDNA tagged at the 3'-end with the sequence encoding a 12-aminoacid peptide CYPYDVPDYASL of influenza hemagglutinin (Snail1 cells) or with an empty vector (Mock cells) were generated as described (Pálmer et al. 2004). Stable ectopic expression of His/Xpress-tagged VDR was achieved in either Snail1 or Mock cells to generate Snail1 + VDR and Mock + VDR cells. The cells were transfected with a vector expressing human VDR (pcDNA3.1 + VDR) using the jetPEI transfection reagent (Polyplus transfection, Illkirch, France) according to the manufacturer's guidelines and selected over 2 weeks with G418 (0.3 mg/ml, Sigma). To generate pcDNA3.1 + VDR, the pRSETA plasmid containing the full-length human *VDR* cDNA cloned in the *BamHI*

site (donated by R Bouillon and G Eelen, University of Leuven, Leuven, Belgium) was digested with *Xba*I and made blunt using DNA polymerase I Klenow fragment. Upon *Xho*I digestion, it was ligated into *Eco*RV/*Xho*I-digested pCDNA3.1 + vector (Invitrogen).

For transient transactivation assays, cells were transfected in triplicate 24-well dishes using the jetPEI transfection reagent. The 4xVDRE-DR3-Tk-Luc construct was provided by C Carlberg (University of Kuopio, Kuopio, Finland). This construct contains four tandem copies of a consensus DR3 response element for vitamin D cloned upstream of the Herpes simplex virus thymidine kinase gene promoter and the luciferase reporter gene. To study β -catenin/TCF transcriptional activity, we transfected cells with either TOP-flash or FOP-flash plasmids provided by H Clevers (Hubrecht Laboratory, Utrecht, The Netherlands). These plasmids contain multimerised wild-type (CCTTTGATC) or mutated (CCTTTGGCC) TCF/LEF-1 binding sites upstream of a minimal *c-fos* promoter to drive luciferase gene expression. Reporter assays were carried out 48 h after transfection by measuring Firefly luciferase (Luc) and Renilla reniformis luciferase (Rluc) activities using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The Luc activity was then normalized to Rluc activity.

All cells were cultured in DMEM supplemented with 10% (v/v) foetal calf serum (FCS; both from Invitrogen). Treatment with $1,25(\text{OH})_2\text{D}_3$ was carried out using DMEM supplemented with charcoal-treated FCS to remove liposoluble hormones. The cells were treated with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ or the corresponding concentration of vehicle/isopropanol for the times indicated. To block endogenous GSK-3 β activity, cells were exposed to LiCl (40 mM, Sigma). Phase-contrast images were captured with a Leica DC 300 digital camera mounted on an inverted Leitz Labovert FS microscope and processed using Adobe Photoshop software.

S.c. tumorigenicity assays

Severe immune-deficient female scid mice were obtained from The Jackson Laboratories (Bar Harbor, ME, USA). Mice were s.c. injected with 5×10^6 Snail1 or Mock-infected SW480-ADH cells in each flank, and treated with EB1089 or placebo as described previously (Pálmer *et al.* 2004). Eight animals were used in each group. The maintenance and handling of animals were as recommended by the European Union (ECC Directive of November 24th, 1986, 86/609/EEC) and all experiments were approved by the Animal Experimentation Committee at our Institute. Every

effort was made to minimize the animal suffering and to reduce the number of animals used.

Western blotting, immunofluorescence and immunohistochemistry

Preparation of whole-cell extracts (Pálmer *et al.* 2001) and cytosolic fractions (González-Sancho *et al.* 2004) have been previously described. The western blots were also performed as described previously (Pálmer *et al.* 2001) and they were probed with antibodies against: β -catenin, ENC-1 and E-cadherin (1/1000, BD Transduction Laboratories, San Diego, CA, USA); c-MYC, EPHB2 and β -actin (1/100, 1/100 and 1/2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA); HA (1/1000, Babco, Richmond, CA, USA); VDR (1/1000, Chemicon, Temecula, CA, USA); p21^{WAF1/CIP1} (1/500, Upstate, Lake Placid, NY, USA); and β -tubulin (1/10000, Sigma). The anti-SNAIL1 antibody (1/20) was generated by ourselves (Rosivatz *et al.* 2006). Immunofluorescence was done as described (Pálmer *et al.* 2001) using the same antibodies against β -catenin (1/100), β -tubulin (1/1000) and SNAIL1 (1/10). Immunostaining of formalin-fixed, paraffin-embedded tumour sections was performed as described (Palacios & Gamallo 1998) using appropriate dilutions of the same anti- β -catenin (1/500) and anti-ENC-1 (1/400) antibodies. 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). Confocal microscopy was performed with a Bio-Rad MRC-1024 laser scanning microscope equipped with a Zeiss Axiovert 100 inverted microscope. All images were processed using Adobe Photoshop software.

Proliferation and migration assays

Cell proliferation was estimated by directly counting the number of trypsinized cells using a Z1 D Coulter Counter at different times following cell treatment with $1,25(\text{OH})_2\text{D}_3$ or vehicle. Each experiment was performed in triplicate. The capacity of cells to migrate was analysed by the wound-healing assay. In this assay, confluent cell monolayers were lightly scratched with a Gilson pipette tip and, after washing to remove detached cells, the cultures were exposed to $1,25(\text{OH})_2\text{D}_3$ or vehicle and observed and photographed after different time intervals. Phase-contrast images were captured with a Leica DC 300 digital camera mounted on an

inverted Leitz Labovert FS microscope and processed using Adobe Photoshop software.

Results

Snail1 blocks the inhibition of β -catenin gene regulatory activity by $1,25(\text{OH})_2\text{D}_3$

As $1,25(\text{OH})_2\text{D}_3$ counteracts the transcriptional activity mediated by β -catenin/TCF (Pálmer *et al.* 2001) and *Snail1* represses *VDR* gene expression (Pálmer *et al.* 2004), we examined whether *Snail1* could block the

effects of $1,25(\text{OH})_2\text{D}_3$ on nuclear β -catenin signalling. To this end, we analysed this phenomena in SW480-ADH cells that expressed high levels of *Snail1* due to retroviral-mediated gene transfer of the mouse *Snail1* cDNA (*Snail1* cells). Cells that were infected with an empty vector were used as a control (Mock cells; Pálmer *et al.* 2004). The expression of *Snail1* inhibited the redistribution of β -catenin protein from the cell nucleus and cytoplasm to the plasma membrane that was induced by $1,25(\text{OH})_2\text{D}_3$ in Mock-infected cells (Fig. 1A). Thus, $1,25(\text{OH})_2\text{D}_3$ diminished the amount of cytosolic β -catenin protein in Mock cells but not in *Snail1* cells

Figure 1 *Snail1* blocks the redistribution of β -catenin protein and the inhibition of β -catenin/TCF transcriptional activity promoted by $1,25(\text{OH})_2\text{D}_3$. (A) Immunostaining of β -catenin protein in Mock-infected and *Snail1* cells incubated in the presence or absence of $1,25(\text{OH})_2\text{D}_3$ for 48 h. Scale bar: 20 μm . (B) The western blot showing the effect of 48 h $1,25(\text{OH})_2\text{D}_3$ treatment on the cytosolic levels of β -catenin protein in Mock-infected and *Snail1* cells. β -Tubulin protein levels served as a control and the numbers refer to the fold-decrease with respect to untreated cells. (C) Mock-infected and *Snail1* cells were transiently transfected with TOP- or FOP-flash plasmids, and with either a *VDR* expression plasmid or an empty vector (pSG5). The cells were then exposed to $1,25(\text{OH})_2\text{D}_3$ (+) or vehicle (-) for 48 h and the TOP-flash/FOP-flash ratio was calculated for each condition. β -Catenin/TCF activity was represented as the percentage of that found in untreated cells (100%). (D) The western blot analysis of the expression of β -catenin and β -catenin-regulated genes in Mock-infected and *Snail1* cells exposed to $1,25(\text{OH})_2\text{D}_3$ (+) or vehicle (-) for 48 h. β -Tubulin was used as a control and the numbers refer to the fold-decrease with respect to untreated cells (upper panels) or to the change with respect to untreated Mock-infected cells (lower panel).

(Fig. 1B). In accordance with the effect of $1,25(\text{OH})_2\text{D}_3$ and Snail1 on the accumulation of β -catenin in the nucleus, Snail1 expression completely blocked the inhibition of β -catenin/TCF transcriptional activity by $1,25(\text{OH})_2\text{D}_3$ (Fig. 1C). Significantly, transient co-transfection of an exogenous *VDR* gene partially restored the effects of $1,25(\text{OH})_2\text{D}_3$ in Snail1 cells (Fig. 1C).

In Mock-infected cells, the expression of genes activated by β -catenin/TCF was inhibited by $1,25(\text{OH})_2\text{D}_3$, as seen for *c-MYC*, ectodermal-neural cortex-1 (*ENC-1*) and ephrin receptor B2 (*EPHB2*; Fig. 1D). In contrast, this inhibition was no longer observed in Snail1 cells. This relief of inhibition was not due to an effect on β -catenin expression as the total cellular content of this protein remained unaffected by $1,25(\text{OH})_2\text{D}_3$ treatment or Snail1 over-expression (Fig. 1D). Together, these data indicate that the inhibition of $1,25(\text{OH})_2\text{D}_3$ activity by Snail1 maintains the Wnt/ β -catenin signalling pathway activated.

Snail1 reverts the effects of $1,25(\text{OH})_2\text{D}_3$ on cell proliferation and migration

β -Catenin probably promotes cell growth through the induction of *c-MYC*, cyclin D1 and other cell cycle regulatory genes, and cell migration through the induction of *MMP7*, *Nr-CAM* and other genes (Tetsu

& McCormick 1999, van de Wetering *et al.* 2002, Giles *et al.* 2003, Verma *et al.* 2003). We found that Snail1 cells proliferated more slowly than Mock-infected cells, consistent with published data (Vega *et al.* 2004). Moreover, proliferation of the cells expressing Snail1 was not affected by $1,25(\text{OH})_2\text{D}_3$ (Fig. 2A). Indeed, unlike Mock-infected cells, Snail1 cells were also resistant to the inhibitory effects of $1,25(\text{OH})_2\text{D}_3$ on cell migration in wound-healing assays (Fig. 2B).

Snail1 abrogates the inhibition of Wnt/ β -catenin signalling by EB1089 *in vivo*

The $1,25(\text{OH})_2\text{D}_3$ analogue EB1089 inhibits tumour growth in immune-deficient mice injected with Mock-infected, but not with Snail1 SW480-ADH cells (Pálmer *et al.* 2004). Thus, we examined whether Snail1 expression might exert a dominant effect over the influence of EB1089 on β -catenin signalling *in vivo* by analysing the distribution of β -catenin in these tumour xenografts. While in untreated mock-infected cells β -catenin was predominantly located in the nucleus and to a lesser extent in the cytosol, only very weak nuclear staining was observed in EB1089-treated animals (Fig. 3, upper left panels). In contrast, Snail1 tumour cells showed strong nuclear (and also cytosolic) β -catenin staining in both untreated and EB1089-treated animals

Figure 2 Snail1 abrogates the inhibition of cell proliferation and migration by $1,25(\text{OH})_2\text{D}_3$. (A) Proliferation of untreated or $1,25(\text{OH})_2\text{D}_3$ -treated Mock-infected and Snail1 cells. (B) Wound healing assay to determine the migratory capacity of Mock-infected and Snail1 cells. Phase-contrast micrographs were taken at the indicated periods of incubation in the presence of $1,25(\text{OH})_2\text{D}_3$ or vehicle upon monolayer scratching. Scale bar, 150 μm .

Figure 3 Snail1 blocks the redistribution of β -catenin and the inhibition of its gene regulatory activity induced by EB1089 *in vivo*. Representative sections of tumours grown in mice injected with Mock-infected or Snail1 cells and treated or not with EB1089, that were immunostained with antibodies against β -catenin and ENC-1. Scale bar, 65 μ m.

(Fig. 3, upper right panels). Accordingly, the protein encoded by the *ENC-1* gene, a target of β -catenin/TCF, accumulates in Snail1 tumour cells irrespective of whether they were exposed to EB1089, while its accumulation in Mock-infected cells diminished upon exposure to this compound (Fig. 3, lower panels).

The induction of endogenous SNAIL1 represses VDR expression and inhibits the transcriptional activity of 1,25(OH)₂D₃

We investigated whether the effects of exogenous Snail1 could be reproduced by augmenting the expression of endogenous SNAIL1. Exposure of the cells to LiCl inhibits GSK-3 β thereby inducing Snail1 transcription as well as inhibiting Snail1 protein degradation and nuclear export (Zhou *et al.* 2004, Bachelder *et al.* 2005, Yook *et al.* 2005). Treatment with LiCl altered the morphology of SW480-ADH cells in a similar manner to the exogenous expression of Snail1. Thus, exposure to LiCl led to an increase in cell size and the cells adopted a stellar shape with large cytoplasmic extensions (compare Fig. 4A with Fig. 1A and also Pálmer *et al.* 2004). Immunofluorescence analysis shows the cellular redistribution of β -tubulin and the elevation of SNAIL1 levels induced by LiCl. The western blot analysis confirmed that the presence of LiCl induced the stable accumulation of SNAIL1 protein and the concomitant reduction in VDR levels (Fig. 4B).

The responsiveness of the cells to 1,25(OH)₂D₃ was studied by analysing the activation of a consensus VDRE and through the regulation of endogenous target genes. Addition of LiCl inhibited VDRE activation (50%, Fig. 4C) and reduced the

accumulation of E-cadherin and p21^{WAF1/CIP1} as well as the inhibition of c-MYC upon exposure to 1,25(OH)₂D₃ (Fig. 4D).

Re-introduction of VDR into Snail1-expressing cells restores their responsiveness to 1,25(OH)₂D₃ but only partially normalizes its inhibitory effect on the Wnt/ β -catenin pathway

To examine to which extent the repression of VDR was responsible for the inhibitory effect of Snail1 on 1,25(OH)₂D₃ activity, we stably expressed an exogenous *VDR* gene in both Snail1 and Mock cells. The expression of this exogenous *VDR* gene did not produce any visible change in cell morphology in either case. In response to 1,25(OH)₂D₃, Snail1 cells ectopically expressing VDR (Snail1 + VDR cells) showed significant stabilization of the VDR protein and the induction of the p21^{WAF1/CIP1} gene, two hallmarks of 1,25(OH)₂D₃ activity (Fig. 5A; Wiese *et al.* 1992, Arbour *et al.* 1993, Liu *et al.* 1996, Saramäki *et al.* 2006). Likewise, ectopic VDR expression restored the transcriptional response of a consensus VDRE to this hormone (Fig. 5B). These data indicate that Snail1 does not affect VDR protein stability or function.

Snail1 + VDR cells only displayed a partial inhibition of β -catenin/TCF transcriptional activity upon exposure to 1,25(OH)₂D₃ (Fig. 6A). Likewise, they showed no inhibition by the hormone of c-MYC and *ENC-1*, two genes induced by β -catenin/TCF (Fig. 6B). Additionally, the blockage by Snail1 of the induction of E-cadherin by 1,25(OH)₂D₃ was barely alleviated by exogenous VDR expression in these cells (Fig. 6B). Further indicating that ectopic VDR is not

Figure 4 The induction of endogenous SNAIL1 reduces VDR expression and impairs 1,25(OH) $_2$ D $_3$ activity in SW480-ADH cells. (A) Phase-contrast micrographs (upper panels) and confocal immunofluorescence images (middle and lower panels) of SW480-ADH cells in the presence or absence of LiCl (10 h). Arrows indicate the appearance of large, stellate cells in LiCl-treated cultures. Scale bar, 40 μ m (upper panels) and 10 μ m (middle and lower panels). (B) The western blot analysis of the expression of SNAIL1 and VDR in SW480-ADH cells maintained in the presence (+) or absence (–) of LiCl for the times indicated. β -Actin was used as a control and the numbers refer to the fold-change with respect to untreated cells. (C) Effect of LiCl on the activation of a consensus VDRE. SW480-ADH cells transiently transfected with 4xVDRE-DR3-Tk-Luc plasmid were pre-treated or not with LiCl for 5 h and then incubated with 1,25(OH) $_2$ D $_3$ or vehicle for an additional 8 h. The induction of a consensus VDRE by 1,25(OH) $_2$ D $_3$ in the presence or absence of LiCl is shown. (D) The western blot showing the effect of LiCl on the induction of E-cadherin and p21^{WAF1/CIP1} and the repression of c-MYC by 1,25(OH) $_2$ D $_3$. Total cell lysates were obtained from SW480-ADH cells pre-treated or not with LiCl for 5 h and incubated with 1,25(OH) $_2$ D $_3$ or vehicle for an additional 12 h. β -Actin was used as a control and the numbers refer to the fold-increase (upper panels) or decrease (lower panel) with respect to vehicle-treated cells.

sufficient to block the effect of Snail1 on Wnt signalling, we found only a partial inhibition of Snail1 + VDR cell proliferation (Fig. 6C) and migration (Fig. 6D) by 1,25(OH) $_2$ D $_3$. Ectopic VDR expression in Mock cells (Mock + VDR cells) did not modify the sensibility to 1,25(OH) $_2$ D $_3$ (Figs 5 and 6).

Discussion

Aberrant activation of the Wnt/ β -catenin signalling pathway resulting from the early mutation of either *APC* or *CTNNB1* genes is commonly found in colon cancer and also in a proportion of other carcinomas (Giles *et al.* 2003, review). The analysis of mice harbouring natural mutations in *Apc* or conditionally expressing mutant β -catenin reinforces the finding that

abnormal activation of the Wnt/ β -catenin pathway is the principal driver of intestinal tumours (Sancho *et al.* 2004, review). Accordingly, significant efforts have been directed towards identifying antagonists of the β -catenin/TCF complex with suitable pharmacological features (Lepourcelet *et al.* 2004). Inhibition of nuclear β -catenin/TCF transcriptional activity restores the differentiated phenotype of colon epithelial cells both *in vitro* and *in vivo* (Mariadason *et al.* 2001, Naishiro *et al.* 2001, van de Wetering *et al.* 2002). A number of pro-oncogenic factors promote the nuclear translocation of β -catenin, whereas anti-oncogenic factors such as activated nuclear receptors inhibit β -catenin signalling (Wong & Pignatelli 2002, Shah *et al.* 2003). In line with this, we previously reported that VDR activated by 1,25(OH) $_2$ D $_3$ or several analogues

Figure 5 Ectopic VDR expression restores the gene regulatory effects of 1,25(OH)₂D₃ in Snail1-infected cells. (A) The western blot showing the expression of Snail1-HA, exogenous and endogenous VDR, and p21^{WAF1/CIP1} in Mock, Snail1, Snail1 + VDR and Mock + VDR cells upon exposure to 1,25(OH)₂D₃ (+) or vehicle (–) for 48 h. β-Actin was used as a control and the numbers refer to the fold-change with respect to endogenous VDR levels in vehicle-treated Mock-infected cells (middle panel) or to the increase with respect to vehicle-treated cells (lower panel). (B) Mock, Snail1, Snail1 + VDR and Mock + VDR cells were transiently transfected with 4xVDRE-DR3-Tk-Luc plasmid and exposed to 1,25(OH)₂D₃ or vehicle for 48 h. Values correspond to VDRE induction by 1,25(OH)₂D₃.

inhibits the Wnt/β-catenin pathway and thus has anti-proliferative and pro-differentiation effects in a series of human colon cancer cell lines (Pálmer *et al.* 2001). This activity is at least in part due to the ligand-dependent interaction between VDR and β-catenin (Pálmer *et al.* 2001). Moreover, the interaction between VDR and β-catenin has recently been confirmed and characterized in detail by others (Shah *et al.* 2006).

Snail1 represses the expression of human *VDR* gene (Pálmer *et al.* 2004) and our results now indicate that Snail1 also impedes the nuclear export of β-catenin induced by 1,25(OH)₂D₃ and its analogues both *in vitro* and *in vivo*. Consequently, Snail1 abrogates

the inhibitory effect of 1,25(OH)₂D₃ on the gene regulation by β-catenin/TCF complexes, as well as, on cell proliferation and migration. Our results point to Snail1 as a positive regulator of β-catenin signalling and reveal a novel action of Snail1 in tumorigenesis: the blockage of the inhibitory effect of 1,25(OH)₂D₃ on the Wnt/β-catenin pathway. Recently, GSK-3β has been shown to diminish the accumulation of Snail1 RNA and protein and to promote its nuclear export (Zhou *et al.* 2004, Bachelder *et al.* 2005, Yook *et al.* 2005). Wnt ligands inhibit GSK-3β and therefore, intensify the presence of Snail1 in the nucleus (Yook *et al.* 2005). Our data indicate that up-regulation of Snail1 in cancer cells may constitute a positive feedback mechanism to maintain the Wnt/β-catenin pathway active.

High VDR expression is associated with a favourable prognosis in colorectal cancer (Cross *et al.* 1996, Evans *et al.* 1998), and both 1,25(OH)₂D₃ and its analogues inhibit tumour promotion by secondary bile acids and tumour load in *Apc*^{min} mice (Huerta *et al.* 2002, Makishima *et al.* 2002). Therefore, an increase in Snail1 expression during colorectal cancer progression may shield tumour cells from the protective effects of 1,25(OH)₂D₃. Our results demonstrate that an increase in Snail1 levels either by expression of an exogenous gene or by the induction of endogenous SNAIL1 abrogates the regulatory effects of 1,25(OH)₂D₃ on gene expression. As Snail1 is induced during the malignant conversion of tumour cells at the adenoma-to-carcinoma transition, it is a candidate to maintain the β-catenin regulatory pathway active during the late stages of cancer progression.

While the re-introduction of VDR into cells expressing Snail1 fully restores the gene regulatory activity of 1,25(OH)₂D₃, there is only partially recovery of the antagonism that this hormone exerts on the Wnt/β-catenin pathway, indicating that both activities act independently. In agreement with this, certain mutations in the carboxy-terminal activation function-2 domain of VDR block its capacity to activate a VDRE but still permit it to interact with β-catenin (Shah *et al.* 2006). Our results show that in addition to the repression of VDR, Snail1 partially overrides the antagonist effect of 1,25(OH)₂D₃-activated VDR on Wnt/β-catenin signalling. Why ectopic VDR expression does not fully restore the inhibition of the Wnt/β-catenin pathway by 1,25(OH)₂D₃ in cells expressing Snail1 is unclear and it is probably due to several reasons. VDR re-expression would be expected to restore the inhibition based on the formation of VDR/β-catenin complexes that interfere with β-catenin/TCF activity. However, the complete inhibition of the pathway by 1,25(OH)₂D₃ may require

Figure 6 Ectopic VDR expression in Snail1-infected cells only partially normalizes the inhibitory effect of 1,25(OH)₂D₃ on Wnt/β-catenin signalling. (A) Effect on β-catenin/TCF transcriptional activity. Mock, Snail1, Snail1 + VDR and Mock + VDR cells transiently transfected with TOP-flash or FOP-flash plasmids were exposed to 1,25(OH)₂D₃ (+) or vehicle (–) for 48 h. TOP-flash/FOP-flash ratio was calculated for each condition and the percentage with respect to vehicle-treated cells is shown. (B) The western blot of c-MYC, ENC-1 and E-cadherin expression in Mock, Snail1, Snail1 + VDR and Mock + VDR cells exposed to 1,25(OH)₂D₃ (+) or vehicle (–) for 48 h. β-Actin was used as a control and the numbers refer to the fold-decrease (upper panels) or increase (lower panel) with respect to untreated cells. (C) Effects of 4-day exposure to 1,25(OH)₂D₃ on the proliferation of Mock-infected, Snail1, Snail1 + VDR and Mock + VDR cells. The percentage values with respect to those obtained from vehicle-treated cells are shown. (D) Wound healing assay to assess the migratory capacity of Mock, Snail1, Snail1 + VDR and Mock + VDR cells. Phase-contrast micrographs were taken at the times indicated following monolayer scratching in the presence of 1,25(OH)₂D₃ or vehicle. Scale bar, 150 μm.

the stable redistribution of β-catenin from the nucleus to the plasma membrane as a consequence of E-cadherin induction by the hormone (Pálmer *et al.* 2001). The weaker induction of E-cadherin in Snail1 + VDR cells

than in Mock or Mock + VDR cells may explain the incomplete redistribution of β-catenin and thus, the partial inhibition of the Wnt/β-catenin signalling pathway.

This poor induction of E-cadherin by $1,25(\text{OH})_2\text{D}_3$ in *Snail1* + VDR cells is most probably due to *Snail1* mediated repression of the *CDH1*/E-cadherin gene promoter. This repression seems to predominate over the induction by ligand-activated VDR. In addition, these effects may depend on *Snail1* regulating a large number of genes involved in epithelial differentiation and cell adhesion, as shown by large-scale gene expression analysis (De Craene *et al.* 2005) and other studies (Barrallo-Gimeno & Nieto 2005, review). Presumably, the repression of occludin or claudins by *Snail1*, and the induction of other genes, such as fibronectin or vimentin can affect the induction of a differentiated phenotype and the inhibition of the Wnt/ β -catenin pathway by $1,25(\text{OH})_2\text{D}_3$.

Acknowledgements

We thank M Lafarga and M T Berciano (University of Cantabria, Santander, Spain) for their help with the confocal microscopy analysis; H Clevers (Hubrecht Laboratory, Utrecht, The Netherlands), C Carlberg (University of Kuopio, Kuopio, Finland), R Bouillon and G Eelen (University of Leuven, Leuven, Belgium) for plasmids; and MR Uskokovic (BioXcell, Nutley, New York, USA) and L Binderup (Leo Pharmaceutical Products, Copenhagen, Denmark) for $1,25(\text{OH})_2\text{D}_3$ and EB1089, respectively. N Valle was supported by a contract of the Fundación Científica de la Asociación Española Contra el Cáncer. This study was supported by Grants from Ministerio de Educación y Ciencia (SAF04-01015) and Instituto de Salud Carlos III (FIS03-C03/10) of Spain. We declare that no conflict of interest exists that prejudices the impartiality of the present study.

References

Arbour NC, Prah J & DeLuca HF 1993 Stabilization of the Vitamin D receptor in rat osteosarcoma cells through the action of $1,25$ -dihydroxyvitamin D₃. *Molecular Endocrinology* **7** 1307–1312.

Bachelder RE, Yoon SO, Francí C, García de Herreros A & Mercurio AM 2005 Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial-mesenchymal transition. *Journal of Cell Biology* **168** 29–33.

Barrallo-Gimeno A & Nieto MA 2005 The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* **132** 3151–3161.

Battle E, Sancho E, Francí C, Domínguez D, Monfar M, Baulida J & García de Herreros A 2000 The transcription factor Snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nature Cell Biology* **2** 84–89.

Blanco MJ, Moreno-Bueno G, Sarrió D, Locascio A, Cano A, Palacios J & Nieto MA 2002 Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene* **21** 3241–3246.

Cano A, Pérez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F & Nieto MA 2000 The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nature Cell Biology* **2** 76–83.

Cross HS, Bajna E, Bises G, Genser D, Kállay E, Pötzi R, Wenzl E, Wrba F, Roka R & Peterlik M 1996 Vitamin D receptor and cytokeratin expression may be progression indicators in human colon cancer. *Anticancer Research* **16** 2333–2337.

De Craene B, Gilbert B, Stove C, Bruyneel E, van Roy F & Berx G 2005 The transcription factor Snail induces tumour cell invasion through modulation of the epithelial cell differentiation program. *Cancer Research* **65** 6237–6244.

Evans SRT, Nolla J, Hanfelt J, Shabahang M, Nauta RJ & Shchepotin IB 1998 Vitamin D receptor expression as a predictive marker of biological behavior in human colorectal cancer. *Clinical Cancer Research* **4** 1591–1595.

Giles RH, van Es JH & Clevers H 2003 Caught up in a Wnt storm: Wnt signalling in cancer. *Biochemical and Biophysical Acta* **1653** 1–24.

González-Sancho JM, Brennan KR, Castelo-Soccio LA & Brown AMC 2004 Wnt proteins induce Dishevelled phosphorylation via an LRP5/6-independent mechanism, irrespective of their ability to stabilize β -catenin. *Molecular and Cellular Biology* **24** 4757–4768.

Grant WB & Garland CF 2004 A critical review of studies on Vitamin D in relation to colorectal cancer. *Nutrition and Cancer* **48** 115–123.

Gumbiner BM 1996 Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84** 345–357.

Hansen CM, Binderup L, Hamberg KJ & Carlberg C 2001 Vitamin D and cancer: effects of $1,25(\text{OH})_2\text{D}_3$ and its analogs on growth control and tumorigenesis. *Frontiers in Bioscience* **6** D820–D848.

Huerta S, Irwin RW, Heber D, Go VLW, Koeffler HP, Uskokovic MR & Harris DM 2002 $1\alpha,25$ -(OH) $_2\text{D}_3$ and its synthetic analogue decrease tumor load in the Apc^{min} mouse. *Cancer Research* **62** 741–746.

Jamora C & Fuchs E 2002 Intercellular adhesion, signalling and the cytoskeleton. *Nature Cell Biology* **4** E101–E108.

Jiao W, Miyazaki K & Kitajima Y 2002 Inverse correlation between E-cadherin and Snail expression in hepatocellular carcinoma cell lines *in vitro* and *in vivo*. *British Journal of Cancer* **86** 98–101.

Lepourcelet M, Chen YN, France DS, Wang H, Crews P, Petersen F, Bruseo C, Wood AW & Shivdasani RA 2004 Small-molecule antagonists of the oncogenic Tcf/ β -catenin protein complex. *Cancer Cell* **5** 91–102.

Liu M, Lee M-H, Cohen M, Bommakanti M & Freedman LP 1996 Transcriptional activation of the Cdk inhibitor p21

- by Vitamin D₃ leads to the induced differentiation of the myelomonocytic cell line U937. *Genes and Development* **10** 142–153.
- Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR & Mangelsdorf DJ 2002 Vitamin D receptor as an intestinal bile acid sensor. *Science* **296** 1313–1316.
- Mariadason JM, Bordonaro M, Aslam F, Shi L, Kuraguchi M, Velcich A & Augenlicht LH 2001 Down-regulation of β -catenin TCF signaling is linked to colonic epithelial cell differentiation. *Cancer Research* **61** 3465–3471.
- Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, Sterner CJ, Notorfrancesco KL, Cardiff RD & Chodosh LA 2005 The transcriptional repressor Snail promotes mammary tumor recurrence. *Cancer Cell* **8** 197–209.
- Naishiro Y, Yamada T, Takaoka AS, Hayashi R, Hasegawa F, Imai K & Hirohashi S 2001 Restoration of epithelial cell polarity in a colorectal cancer cell line by suppression of β -catenin/T-cell factor 4-mediated gene transactivation. *Cancer Research* **61** 2751–2758.
- Ordóñez-Morán P, Larriba MJ, Pendás-Franco N, Aguilera O, González-Sancho JM & Muñoz A 2005 Vitamin D and cancer: an update of *in vitro* and *in vivo* data. *Frontiers in Bioscience* **10** 2723–2749.
- Palacios J & Gamallo C 1998 Mutations in the β -catenin gene (CTNNB1) in endometrioid ovarian carcinomas. *Cancer Research* **58** 1344–1347.
- Pálmer HG, González-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, Quintanilla M, Cano A, García de Herreros A, Lafarga M *et al.* 2001 Vitamin D₃ promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β -catenin signalling. *Journal of Cell Biology* **154** 369–387.
- Pálmer HG, Larriba MJ, García JM, Ordóñez-Morán P, Peña C, Peiró S, Puig I, Rodríguez R, de la Fuente R, Bernad A *et al.* 2004 The transcription factor SNAIL represses Vitamin D receptor expression and responsiveness in human colon cancer. *Nature Medicine* **10** 917–919.
- Peña C, García JM, Silva J, García V, Rodríguez R, Alonso I, Millán I, Salas C, García de Herreros A, Muñoz A *et al.* 2005 E-cadherin and Vitamin D receptor regulation by SNAIL and ZEB1 in colon cancer: clinicopathological correlations. *Human Molecular Genetics* **14** 3361–3370.
- Poser I, Domínguez D, García de Herreros A, Varnai A, Buettner R & Bosserhoff AK 2001 Loss of E-cadherin expression in melanoma cells involves up-regulation of the transcriptional repressor Snail. *Journal of Biological Chemistry* **276** 24661–24666.
- Rosivatz E, Becker I, Specht K, Fricke E, Lubert B, Busch R, Höfler H & Becker K-F 2002 Differential expression of the epithelial-mesenchymal transition regulators Snail, SIP1, and Twist in gastric cancer. *American Journal of Pathology* **161** 1881–1891.
- Rosivatz E, Becker K-F, Kremmer E, Schott C, Blechschmidt K, Höfler H & Sarbia M 2006 Expression and nuclear localization of Snail, an E-cadherin repressor, in adenocarcinomas of the upper gastrointestinal tract. *Virchows Archiv* **448** 277–287.
- Roy HK, Smyrk TC, Koetsier J, Victor TA & Wali RK 2005 The transcriptional repressor SNAIL is overexpressed in human colon cancer. *Digestive Diseases and Sciences* **50** 42–46.
- Saito T, Oda Y, Kawaguchi K, Sugimachi K, Yamamoto H, Tateishi N, Tanaka K, Matsuda S, Iwamoto Y, Ladanyi M *et al.* 2004 E-cadherin mutation and Snail overexpression as alternative mechanisms of E-cadherin inactivation in synovial sarcoma. *Oncogene* **23** 8629–8638.
- Sancho E, Batlle E & Clevers H 2004 Signalling pathways in intestinal development and cancer. *Annual Review of Cell and Development Biology* **20** 695–723.
- Saramäki A, Banwell CM, Campbell MJ & Carlberg C 2006 Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the Vitamin D3 receptor. *Nucleic Acids Research* **34** 543–554.
- Shah S, Hecht A, Pestell R & Byers SW 2003 Trans-repression of β -catenin activity by nuclear receptors. *Journal of Biological Chemistry* **278** 48137–48145.
- Shah S, Islam MN, Dakshanamurthy S, Rizvi I, Rao M, Herrell R, Zinser G, Valrance M, Aranda A, Moras D *et al.* 2006 The molecular basis of Vitamin D receptor and β -catenin crossregulation. *Molecular Cell* **21** 799–809.
- Tetsu O & McCormick F 1999 β -catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398** 422–426.
- van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis A-P *et al.* 2002 The β -catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111** 241–250.
- Vega S, Morales AV, Ocaña OH, Valdés F, Fabregat I & Nieto MA 2004 Snail blocks the cell cycle and confers resistance to cell death. *Genes and Development* **18** 1131–1143.
- Verma UN, Surabhi RM, Schmalstieg A, Becerra C & Gaynor RB 2003 Small interfering RNAs directed against β -catenin inhibit the *in vitro* and *in vivo* growth of colon cancer cells. *Clinical Cancer Research* **9** 1291–1300.
- Wiese RJ, Uhland-Smith A, Ross TK, Prahl JM & DeLuca HF 1992 Up-regulation of the Vitamin D receptor in response to 1,25-dihydroxyvitamin D₃ results from ligand-induced stabilization. *Journal of Biological Chemistry* **267** 20082–20086.
- Wong NACS & Pignatelli M 2002 β -catenin—a linchpin in colorectal carcinogenesis? *American Journal of Pathology* **160** 389–401.
- Yook JI, Li X-Y, Ota I, Fearon ER & Weiss SJ 2005 Wnt-dependent regulation of the E-cadherin repressor Snail. *Journal of Biological Chemistry* **280** 11740–11748.
- Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M & Hung M-C 2004 Dual regulation of Snail by GSK-3 β -mediated phosphorylation in control of epithelial-mesenchymal transition. *Nature Cell Biology* **6** 931–940.