The inhibition of Wnt/β-catenin signalling by 1α,25-dihydroxyvitamin D3 is abrogated by Snail1 in human colon cancer cells

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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. 1α,25-dihydroxyvitamin D3 (1,25(OH)2D3) 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)2D3 in SW480-ADH cells. Accordingly, Snail1 relieves the inhibition exerted by 1,25(OH)2D3 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)2D3 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)2D3 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)2D3. However, this exogenous VDR failed to fully restore the blockage of the Wnt/β-catenin pathway by 1,25(OH)2D3. 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)2D3.

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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). 1α,25-dihydroxyvitamin D3 (1,25(OH)2D3), 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)2D3 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)2D3 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)2D3 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)-3β 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)2D3 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)2D3 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)2D3 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 (Barralillo-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)2D3 on β-catenin nuclear signalling and on the gene expression driven by this pathway. Likewise, Snail1 reverses the effects of 1,25(OH)2D3 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)2D3 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)2D3 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 XbaI and made blunt using DNA polymerase I Klenow fragment. Upon XhoI digestion, it was ligated into EcoRV/XhoI-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 Carlborg (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(OH)2D3 was carried out using DMEM supplemented with charcoal-treated FCS to remove liposoluble hormones. The cells were treated with 10−7 M 1,25(OH)2D3 or the corresponding concentration of vehicle/isopropanol for the times indicated. To block endogenous GSK-3β 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 × 10⁶ Snail1 or Mock-infected SW480-ADH cells in each flank, and treated with EB1089 or placebo as described previously (Pálmé 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 (Pálmé et al. 2001) and cytosolic fractions (González-Sancho et al. 2004) have been previously described. The western blots were also performed as described previously (Pálmé 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); p21WAF1/CIP1 (1/500, Upstate, Lake Placid, NY, USA); and β-tubulin (1/10000, Sigma). The anti-SNAI1 antibody (1/20) was generated by ourselves (Rosivatz et al. 2006). Immunofluorescence was done as described (Pálmé et al. 2001) using the same antibodies against β-catenin (1/100), β-tubulin (1/1000) and SNAI1 (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 Axioskop 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(OH)2D3 or vehicle. Each experiment was performed in triplicate. The capacity of cells to migrate was assessed 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(OH)2D3 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(OH)_{2}D_{3}**

As 1,25(OH)_{2}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(OH)_{2}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(OH)_{2}D_{3} in Mock-infected cells (Fig. 1A). Thus, 1,25(OH)_{2}D_{3} diminished the amount of cytosolic β-catenin protein in Mock cells but not in Snail1 cells.

![Figure 1](https://www.endocrinology-journals.org)

**Figure 1** Snail1 blocks the redistribution of β-catenin protein and the inhibition of β-catenin/TCF transcriptional activity promoted by 1,25(OH)_{2}D_{3}. (A) Immunostaining of β-catenin protein in Mock-infected and Snail1 cells incubated in the presence or absence of 1,25(OH)_{2}D_{3} for 48 h. Scale bar: 20 μm. (B) The western blot showing the effect of 48 h 1,25(OH)_{2}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(OH)_{2}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(OH)_{2}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).
In accordance with the effect of 1,25(OH)2D3 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(OH)2D3 (Fig. 1C). Significantly, transient co-transfection of an exogenous VDR gene partially restored the effects of 1,25(OH)2D3 in Snail1 cells (Fig. 1C).

In Mock-infected cells, the expression of genes activated by β-catenin/TCF was inhibited by 1,25(OH)2D3, 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(OH)2D3 treatment or Snail1 over-expression (Fig. 1D). Together, these data indicate that the inhibition of 1,25(OH)2D3 activity by Snail1 maintains the Wnt/β-catenin signalling pathway activated.

**Snail1 reverts the effects of 1,25(OH)2D3 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(OH)2D3 (Fig. 2A). Indeed, unlike Mock-infected cells, Snail1 cells were also resistant to the inhibitory effects of 1,25(OH)2D3 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(OH)2D3 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.
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-3β 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 p21⁰⁰⁰¹/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 p21⁰⁰⁰¹/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 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 μm.)
sufficient to block the effect of Snail1 on Wnt signalling, we found only a partial inhibition of Snail1 expression and impaired 1,25(OH)2D3 activity in SW480-ADH cells. Ectopic VDR expression in Mock cells (Mock CVDR cells) did not modify the sensitivity to 1,25(OH)2D3 (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)2D3 or several analogues...
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<sup>min</sup> 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 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<sup>WAF1/CIP1</sup> 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.
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.

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.
This poor induction of E-cadherin by 1,25(OH)₂D₃ 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(OH)₂D₃.

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