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The effects of 1,25-dihydroxyvitamin D_3 on colon cancer cells depend on RhoA-ROCK-p38MAPK-MSK signaling☆

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ABSTRACT

Many studies support a protective action of vitamin D against colon cancer. 1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) exerts wide gene regulatory effects in human colon cancer cells. We previously reported that $1,25(OH)_2D_3$ increases cytosolic Ca^{2+} concentration and transiently activates RhoA and its effector the Rho-associated coiled-kinase (ROCK), and later p38MAPK-MSK. We found that the inhibition of ROCK signaling by Y27632 or that of MSK by Ro318220 prevent the formation of epithelioid islands of SW480-ADH cells by $1,25(OH)_2D_3$ and disrupts the adhesive phenotype of HT29 cells. ROCK and MSK inhibition also abrogates the induction of $1,25(OH)_2D_3$ 24-hydroxylase (*CYP24*), E-cadherin, and vinculin and the repression of cyclin D1 by $1,25(OH)_2D_3$. Moreover, $1,25(OH)_2D_3$ does not promote the localization of the tight junction protein occludin at the plasma membrane in cells expressing a dominant negative RhoA (N19-RhoA). In addition, $1,25(OH)_2D_3$ specifically increases the level of the cysteine protease-inhibitor cystatin D, whereas that of cystatin SN is unaffected. The increase of cystatin D protein caused by $1,25(OH)_2D_3$ is abrogated in N19-RhoA cells. Thus, activation of the RhoA-ROCK-p38MAPK-MSK signaling pathway is essential for the regulation of the phenotype and of the *CST5*/cystatin D candidate tumor suppressor and other target genes by $1,25(OH)_2D_3$ in colon cancer cells.

1. Introduction

The active vitamin D metabolite 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and several less calcemic analogs are the subject of extensive study as potential anticancer drugs [1,2]. In particular, epidemiological and preclinical studies suggest a preventive effect of these compounds against colon cancer and possible therapeutic applications [3–6]. Here we study the gene regulatory effects and mechanism of action of $1,25(OH)_2D_3$ in human colon cancer cells and tumors. Previously, we reported that $1,25(OH)_2D_3$ promotes an adhesive epithelial phenotype and inhibits the proliferation of SW480-ADH cells in part *via* the induction of E-cadherin expression and the antagonism of the Wnt/β-catenin signaling pathway [7]. Moreover, $1,25(OH)_2D_3$ radically changes the gene expression profile of SW480-ADH cells: it positively or negatively regulates over 240 genes [8]. Recently, we reported that $1,25(OH)_2D_3$ increases the Ca^{2+} concentration in the cytosol by promoting Ca^{2+} influx from the external medium. We further reported that this effect is followed by the transient activation of the small GTPase RhoA and its effector the Rho-associated coiled-kinase (ROCK), and later of the p38 mitogen-activated protein kinase (p38MAPK) and mitogen- and stress-activated kinase (MSK). This Ca^{2+}-RhoA-ROCK-p38MAPK-MSK signaling cascade is required for the regulation of several target genes and to interfere with the Wnt/β-catenin pathway. Moreover, vitamin D receptor (VDR) knock-down abrogates the induction of non-genomic signaling and gene expression by $1,25(OH)_2D_3$ [9].

In addition, we identified *CST5*/cystatin D as a novel target gene for $1,25(OH)_2D_3$ in colon cancer cells [10]. Cystatin D is an inhibitor of several cysteine proteases in the cathepsin family. $1,25(OH)_2D_3$ increases the level of cellular cystatin D RNA and protein. Unexpectedly, cystatin D inhibits the proliferation, migration and anchorage-independent growth of cultured colon cancer cells and their capacity to generate tumors in immunodeficient mice [10]. Also, cystatin D antagonizes the Wnt/β-catenin pathway and it represses several genes that encode epithelial-to-mesenchymal transition inducers. In human colorectal tumors the expression of

Abbreviations: MAPK, mitogen-activated protein kinase; MSK, mitogen- and stress-activated kinase; ROCK, Rho-associated coiled-kinase; VDR, vitamin D receptor; $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3.

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cystatin D correlates with that of VDR, while cystatin D loss is associated with poor tumor differentiation [10].

We use Y27632 and Ro318220, inhibitors of ROCK and MSK, respectively, to analyze the importance of the Ca^{2+}-RhoA-ROCK-p38MAPK-MSK signaling cascade on the cellular effects of $1,25(OH)_2D_3$. Both compounds prevented the increase of intercellular adhesion induced by $1,25(OH)_2D_3$ in SW480-ADH and HT29 cells. Furthermore, they hampered the regulation of several $1,25(OH)_2D_3$ target genes. Moreover, expression of a dominant negative RhoA (N19-RhoA) inhibited the subcellular redistribution of the tight junction protein occludin and the increase of cystatin D induced by $1,25(OH)_2D_3$.

2. Materials and methods

2.1. Cell culture, reagents, constructs and transfections

SW480-ADH (derived from the SW480 human colon cancer cell line by limit dilution [7]) and HT29 cells were cultured in DMEM plus 10% foetal bovine serum (Invitrogen). All experiments using $1,25(OH)_2D_3$ (provided by R. Bouillon and A. Verstuyf, Katholieke Universiteit, Leuven, Belgium, and J.P. van de Velde, Solvay, Weesp, Netherlands) were performed in DMEM supplemented with charcoal-treated serum to remove liposoluble hormones. The cells were treated with 10^{-7} M $1,25(OH)_2D_3$ or the corresponding vehicle/ethanol for the times indicated. Y27632, SB203580 and Ro318220 were supplied by Calbiochem.

Cells were transfected using jetPEI reagent (PolyPlus Transfection). In transient transfections, *Firefly* (Luc) and *Renilla reniformis* luciferase (Rluc) activities were measured separately using the Dual Luciferase reagent kit (Promega) and a Lumat LB9507 luminometer (Berthold). Luc activity was normalized to the Rluc activity. Several fragments of the *CST5*/cystatin D promoter (−1867/+262, −1128/+262, −650/+262 and −251/+262) were amplified as described elsewhere [10]. The RhoA constructs were obtained from P. Crespo (Universidad de Cantabria, Santander, Spain). Mock and N19-RhoA cells were generated by stable transfection of SW480-ADH cells with HA-tagged empty vector or HA-N19-RhoA cDNA and selected with G418 (0.3 mg/ml, Sigma) as described elsewhere [9].

2.2. Gene silencing

To knock-down *VDR* and *CST5* cells were infected with lentiviral particles as described elsewhere [9,10].

2.3. Cell proliferation assays

The cells (8×10^3) were seeded in 24-well plates and treated for the indicated times with 10^{-7} M of $1,25(OH)_2D_3$ or vehicle. Cell proliferation was measured by [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) assays that were performed following the manufacturer's instructions (Roche Diagnostics). Experiments were performed in quadruplicate.

2.4. Western blotting

Whole-cell extracts were prepared by washing the monolayers twice in phosphate-buffered saline (PBS) and cell lysis by incubation in RIPA buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 10% glycerol, 4 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 50 mM Hepes pH 7.4) plus phosphatase- and protease-inhibitor mixture (25 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 15 min on ice followed by centrifugation at 13,000 rpm for 10 min at 4 °C. Protein concentration was measured using the Bio-Rad DC protein assay kit. Analysis of cell lysates was performed by electrophoresis in SDS gels and protein transfer to Immobilon P membranes (Millipore Corp.). The membranes were incubated with the appropriate primary and secondary horseradish peroxidase-conjugated antibodies, and the antibody binding was visualized using an ECL detection system (Amersham-G.E. Healthcare). We used rabbit polyclonal antibodies generated against cystatin D [11] and cyclin D1 (Santa Cruz Biotechnology), mouse monoclonal antibodies against E-cadherin (BD) and cystatin SN (R&D Systems); and goat polyclonal antibodies against vinculin, β-actin and lamin B (Santa Cruz Biotechnology) and cystatin C (R&D Systems). The secondary antibodies used were HPR-conjugated anti-rabbit IgG (H + L) (MP Biomedicals), anti-mouse IgG (H + L) (Promega), anti-rat IgG and anti-goat IgG (Santa Cruz Biotechnology). Conditioned media (CM) were obtained basically as described elsewhere [12]. Cells grown to 80% confluency were then incubated in serum-free medium for an additional 48 h. CM were harvested, centrifuged for 10 min at $2000 \times g$, and concentrated 100-fold using Amicon Ultra-15 and Microcon YM-10 columns (Millipore).

2.5. Immunofluorescence and confocal microscopy

Cells were rinsed once in PBS and fixed in 3.7% formaldehyde for 10 min at room temperature. The cells were permeabilized in 0.2% Triton X-100 for 10 min at room temperature. Non-specific sites were blocked by incubation with PBS containing 1% Difco™ Skimmed Milk (BD) for 10 min at room temperature before incubating the cells with anti-occludin antibody (Zymed) (1/100 dilution) and rabbit polyclonal cystatin D antibody (1/100) in PBS for 1 h at 37 °C. After four washes in PBS, the cells were incubated with Alexa 488 goat anti-rabbit (Molecular Probes-Invitrogen) for 45 min at room temperature, washed three times in PBS and mounted in VectaShield (Vector Laboratories). Images were obtained using an Olympus DP70 digital camera mounted on a Zeiss Axiophot microscope equipped with epifluorescence and confocal images were captured with a Leica TCS SP2 confocal microscope. Phase-contrast images were captured with a Leica DC300 digital camera mounted on an inverted Leitz Labovert FS Microscope. All the images were processed using Adobe Photoshop CS2 software.

2.6. Real-time reverse transcription (RT)-PCR

Total RNA was purified using RNeasy mini kits following the manufacturer's instructions (QIAGEN), cDNA synthesis and the PCR conditions were as described elsewhere [9]. The level of *CYP24* RNA was measured in relation to that of *18S* rRNA using the comparative C_T method and RNA TaqMan probes (Applied Biosystems). RNA levels for *OPN*/osteopontin and *OCN*/osteocalcin, and levels of *GAPDH*/glyceraldehyde-3-phosphate dehydrogenase used as a control, were measured as described elsewhere [9].

2.7. Statistical analysis

Data are expressed as the mean ± SD unless otherwise specified. Statistical significance was assessed by two-tailed unpaired student's *t*-test. The single asterisk indicates $P < 0.05$, the double asterisk $P < 0.01$, and the triple asterisk $P < 0.001$. When $P > 0.05$, the data were considered not significant (ns). All statistical analyses were performed using SPSS 13.0 statistical software (SPSS Inc.).

3. Results

3.1. RhoA-ROCK and p38MAPK-MSK signaling is necessary for the gene regulatory action of $1,25(OH)_2D_3$

To study the importance of the RhoA-ROCK-p38MAPK-MSK signaling cascade for the phenotype of human colon cancer cells

we first used Y27632 and Ro318220, inhibitors of ROCK and MSK, respectively. In line with previous results [9], both Y27632 and Ro318220 promoted an elongated morphology in untreated SW480-ADH cells and prevented the formation of compact epithelioid islands following $1,25(OH)_2D_3$ addition (Fig. 1A, left). In the more differentiated HT29 cells these two compounds also partially disrupted the adhesive phenotype in the absence or presence of $1,25(OH)_2D_3$ (Fig. 1A, right). Concordantly with these effects, pretreatment of SW480-ADH cells with Ro318220 strongly reduced the induction by $1,25(OH)_2D_3$ of E-cadherin protein, a crucial component of *adherens junctions* and largely responsible for intercellular adhesion in epithelial cells (Fig. 1B). Likewise, pretreatment with Y27632 of HT29 cells countered the increase in E-cadherin, and also that in vinculin by $1,25(OH)_2D_3$ (Fig. 1C). Moreover, Y27632 abrogated the reduction of cyclin D1 protein caused by $1,25(OH)_2D_3$ (Fig. 1C). The expression of the *CYP24* gene, which encodes the enzyme that catabolizes $1,25(OH)_2D_3$ and is most responsive to $1,25(OH)_2D_3$ induction, was also studied. Y27632 (Fig. 1D), and both Ro318220 and the p38MAPK inhibitor SB203580 (Fig. 1E) lessened the increase in *CYP24* RNA in HT29 cells.

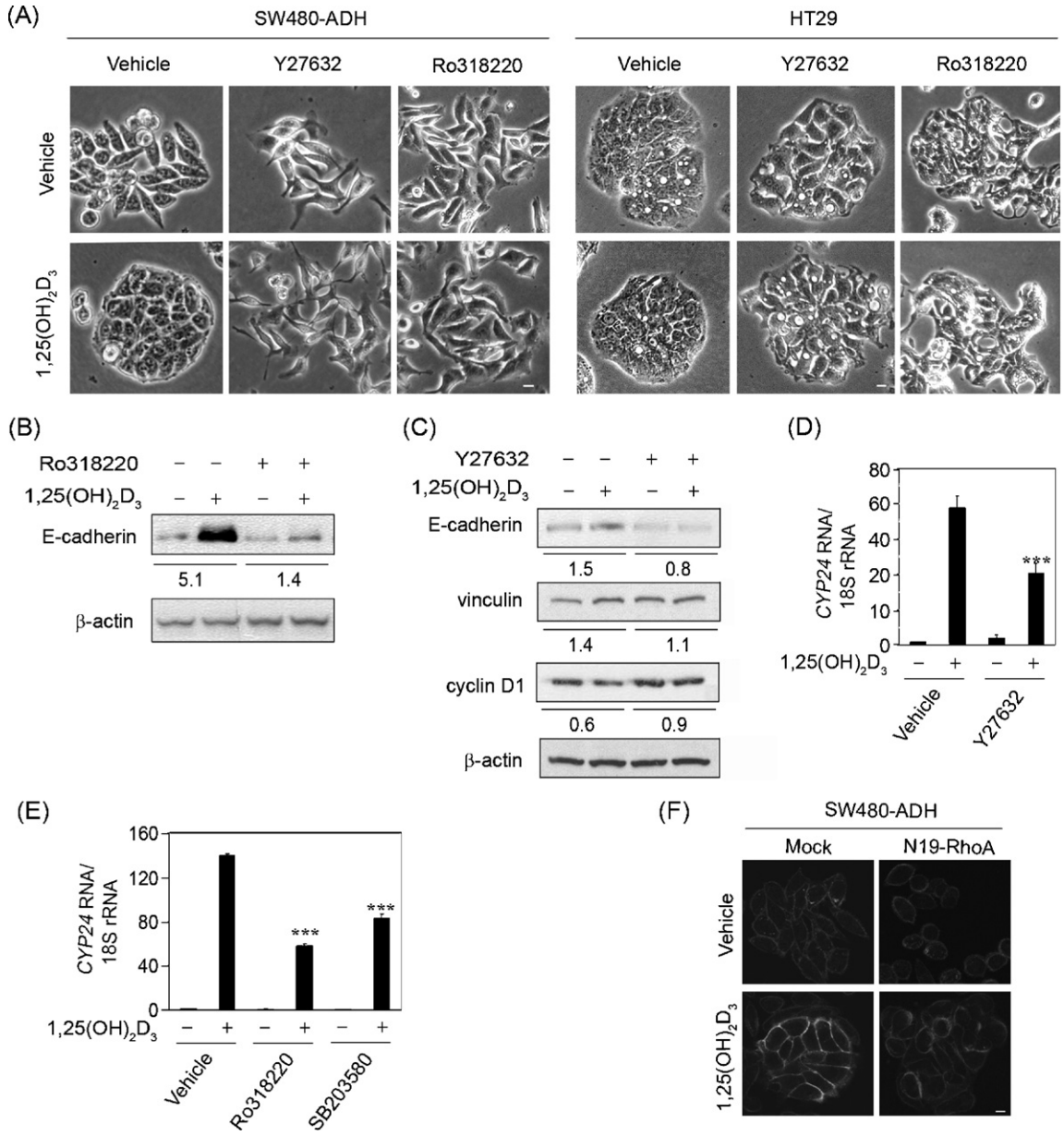

Fig. 1. Activation of RhoA and MSK is required for $1,25(OH)_2D_3$ effects on the phenotype and target genes in human colon cancer cells. (A) Phase-contrast images of cells pretreated with Y27632 (10 μM) or Ro318220 (1 μM) or vehicle for 4 h and then incubated with $1,25(OH)_2D_3$ (10^{-7} M) or vehicle for an additional 48 h. Bar, 10 μm. (B) Lysates of SW480-ADH cells pretreated with Ro318220 (1 μM) or vehicle for 2 h and treated with $1,25(OH)_2D_3$ (10^{-7} M) or vehicle for 48 h, were analyzed by Western blotting with antibodies against E-cadherin or β-actin. The numbers below tracks represent the fold-increase. (C) Lysates of HT29 cells pretreated with Y27632 (10 μM) or vehicle for 4 h and then treated with $1,25(OH)_2D_3$ (10^{-7} M) or vehicle for 48 h, were analyzed by Western blotting with antibodies against E-cadherin, vinculin, cyclin D1, or β-actin. The numbers below tracks represent the fold-increase. (D) RNAs extracted from HT29 cells pretreated with Y27632 (10 μM) or vehicle for 4 h and then treated with $1,25(OH)_2D_3$ (10^{-7} M) or vehicle for 48 h, were analyzed by qRT-PCR with probes for *CYP24* and *18S* RNA. (E) RNAs extracted from HT29 cells pretreated with Ro318220 (1 μM) or SB203580 (20 μM) or vehicles for 2 h and then treated with $1,25(OH)_2D_3$ (10^{-7} M) or vehicle for 48 h, were analyzed by qRT-PCR with probes for *CYP24* and *18S* RNA. (F) Confocal microscopy and immunofluorescence analysis of occludin expression in mock and N19-RhoA SW480-ADH cells cultured in the presence or absence of $1,25(OH)_2D_3$ (10^{-7} M) for 48 h. Bar, 10 μm.

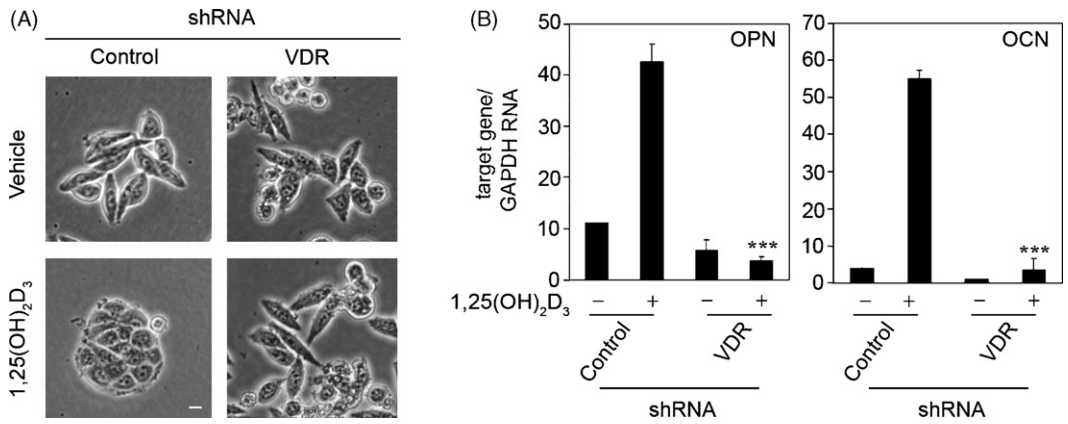

Fig. 2. VDR silencing affects cell morphology and abrogates the induction of *OPN*/osteopontin and *OCN*/osteocalcin by $1,25(OH)_2D_3$. (A) Phase-contrast images of control and VDR shRNA SW480-ADH cells cultured in the presence or absence of $1,25(OH)_2D_3$ (10^{-7} M) for 48 h. Bar, 10 μm. (B) RNAs extracted from cells treated with $1,25(OH)_2D_3$ (10^{-7} M) or vehicle for 48 h were analyzed by qRT-PCR with probes for *OPN*/osteopontin or *OCN*/osteocalcin RNA. *GAPDH* RNA was used as a control.

We previously reported the inhibition of gene regulation by $1,25(OH)_2D_3$ in SW480-ADH cells stably expressing a dominant negative mutant RhoA (N19-RhoA) [9]. In accordance with this, we observed that the induction by $1,25(OH)_2D_3$ of the tight junction protein occludin and its relocalization at the plasma membrane is blocked in N19-RhoA cells (Fig. 1F).

The increase in cytosolic Ca^{2+} concentration and also the activation of RhoA, p38MAPK, and MSK by $1,25(OH)_2D_3$ depend on VDR expression [9]. Indeed, the phenotype of cells in which VDR was silenced by means of short hairpin (sh)RNA did not change after $1,25(OH)_2D_3$ treatment (Fig. 2A). We also found that the induction of *OPN*/osteopontin and *OCN*/osteocalcin genes by $1,25(OH)_2D_3$ is greatly abrogated in VDR knock-down cells. (Fig. 2B).

3.2. *CST5*/cystatin D induction by $1,25(OH)_2D_3$ is specific and requires RhoA activity

Recently, $1,25(OH)_2D_3$ has been shown to induce the expression of *CST5*/cystatin D in SW480-ADH cells [10]. We wished to examine whether this effect was specific or by contrast part of a more general regulatory effect on other members of the cystatin family. By Western blotting we found that $1,25(OH)_2D_3$ increases the level of cystatin D protein in SW480-ADH cells but does not change that of cystatin SN (Fig. 3A). Although the induction of cystatin D by $1,25(OH)_2D_3$ is mainly observed in the intracellular pool, it was also found to a lesser degree in the secreted pool (Fig. 3B). No changes were found in the case of cystatin C (cystatins SN and C were studied, respectively in cell lysates and conditioned medium due to their preferential location in these compartments). Immunofluorescence analysis confirmed the induction of cystatin D in SW480-ADH and HCT116 colon cancer cells (Fig. 3C).

Transactivation assays in SW480-ADH cells revealed that $1,25(OH)_2D_3$ activates the *CST5*/cystatin D promoter probably through several consensus VDR binding sites located in the proximal region (Fig. 3D).

The importance of *CST5*/cystatin D upregulation for $1,25(OH)_2D_3$ action was analyzed by its ectopic expression in the absence of $1,25(OH)_2D_3$ treatment. Exogenous cystatin D changed the level of adhesion proteins and the expression of epithelial-to-mesenchymal transition inducers, and promoted alterations in cell morphology, proliferation and migration capacity [10]. In addition and in concordance with these results, *CST5*/cystatin D knock-down using shRNA abolished the reduction of SW480-ADH cell proliferation in the presence of $1,25(OH)_2D_3$ (Fig. 3E). Interestingly, the level of cystatin D expression decreases during the progression of human colon cancer associated with tumor dedifferentiation ([10] and Fig. 3F).

We sought to elucidate whether the induction of *CST5*/cystatin D requires the activation of the RhoA-ROCK-p38MAPK-MSK signaling cascade. To this end, cystatin D protein expression was studied in mock and in N19-RhoA SW480-ADH cells that had been treated with $1,25(OH)_2D_3$ or vehicle. The increase of cystatin D protein expression by $1,25(OH)_2D_3$ was abrogated in cells that expressed N19-RhoA (Fig. 4A).

4. Discussion

The protective action of $1,25(OH)_2D_3$ and related compounds against colon cancer suggested by epidemiological and experimental studies using cultured cell and animals are believed to be due to their profound effects on gene expression. Thus, $1,25(OH)_2D_3$ exerts a wide gene regulatory action inducing or repressing a high number of genes in different types of cell, including human colon cancer cells [8,13]. Such broad regulation is achieved through binding to VDR, which acts as a ligand-modulated transcription factor by forming heterodimers with RXR that bind to, and control the transcription rate of their target genes [14]. However, VDR is now known to have a more complex mode of action than had been thought for years. Recent data indicate that in human colon cancer cells, and also in other cell types, VDR triggers rapid extranuclear signaling cascades such as that initiated by Ca^{2+} influx which leads to the transient activation of RhoA-ROCK and the kinases p38MAPK and MSK [9]. In addition, VDR may also affect gene transcription by interacting with other proteins such as the DNA-bound VDR-interacting repressor (VDIR) or with β-catenin (for a review see [15]). Our results show that the activation of the two modules RhoA-ROCK and p38MAPK-MSK is indeed essential for the regulatory action of $1,25(OH)_2D_3$ on genes that encode crucial adhesion proteins (E-cadherin, occludin, vinculin), cell cycle regulators (cyclin D1) or *CYP24*, gene that encodes the enzyme responsible for its own catabolism (Fig. 4B).

By using chemical inhibitors and by expressing a dominant negative mutant RhoA, we found that the RhoA-ROCK-p38MAPK-MSK signaling cascade is required for the modulation of gene expression by $1,25(OH)_2D_3$. As knock-down experiments have shown that VDR is necessary for both the early signaling and the transcriptional effects, this member of the nuclear receptor superfamily is a mediator of $1,25(OH)_2D_3$ with both extranuclear/non-genomic and nuclear/genomic activity. However, the exact mechanism by

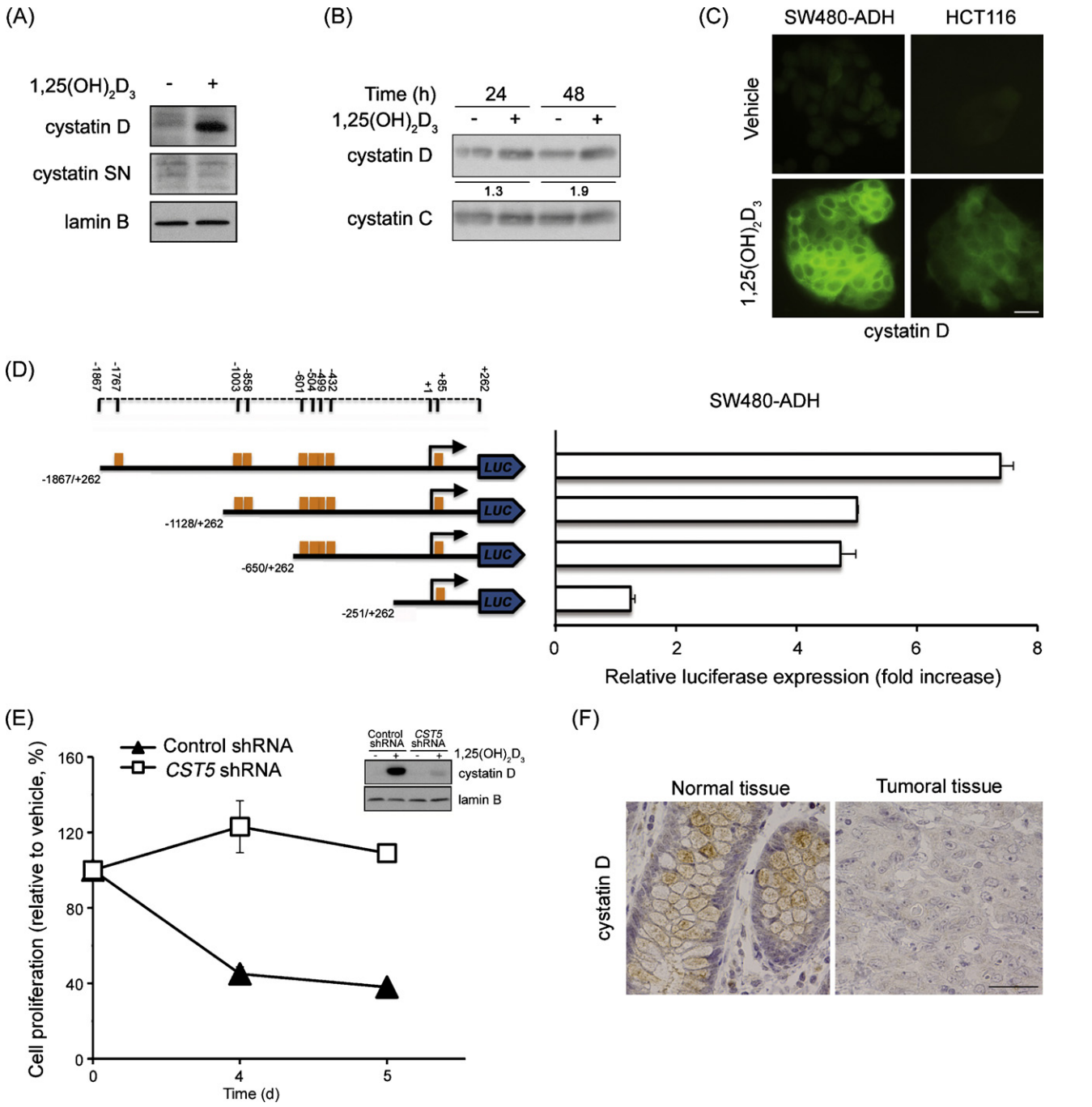

Fig. 3. *CST5*/cystatin D is specifically induced by $1,25(OH)_2D_3$ at the transcription level and mediates its antiproliferative effect on SW480-ADH cells. (A) Lysates of cells treated or not with 10^{-7} M $1,25(OH)_2D_3$ for 48 h were analyzed by Western blotting with antibodies against cystatin D or cystatin SN. Lamin B was used as a loading control. (B) Conditioned media of cells treated or not with 10^{-7} M $1,25(OH)_2D_3$ for 24 or 48 h were analyzed by Western blotting with antibodies against cystatin D or cystatin C. The numbers below tracks represent the fold-increase. (C) Confocal microscopy and immunofluorescence analysis of cystatin D expression in cells treated with $1,25(OH)_2D_3$ (10^{-7} M) or vehicle for 48 h. Bar, 20 μm. (D) Activation of a series of *CST5*/cystatin D promoter constructs that were transfected into SW480-ADH cells that were treated for 48 h with $1,25(OH)_2D_3$ (10^{-7} M). Boxes in the promoter indicate putative consensus VDR binding sites [10]. (E) Cell proliferation was measured by the MTT assay. *CST5*/cystatin D silencing was checked by Western blotting analysis of cell lysates using antibodies against cystatin D and lamin B (inset). (F) Representative image of the immunohistochemical analysis of cystatin D expression in normal and de-differentiated tumoral tissue from a colon cancer patient. Bar, 100 μm.

which VDR activates the extranuclear signaling remains unknown, as does its precise location outside the cell nucleus. Although it has not been detected in colon cancer cells, some VDR molecules may reside at the plasma membrane or, alternatively, transiently translocate there from cytoplasmic reservoir(s) upon passive entry of $1,25(OH)_2D_3$ to this compartment [16]. Such a VDR population may be responsible, directly or indirectly, for the modulation of Ca^{2+} channels that initiate the signaling pathway. VDR is required for both the early activation of the extranuclear signaling cascade and later for the regulation of target gene transcription [9]. Thus, VDR has a dual action as a transcription factor and a non-genomic activator of RhoA-ROCK and p38MAPK-MSK.

Among the genes that are regulated by $1,25(OH)_2D_3$ in human colon cancer cells, *CST5*/cystatin D seems to be particularly impor-

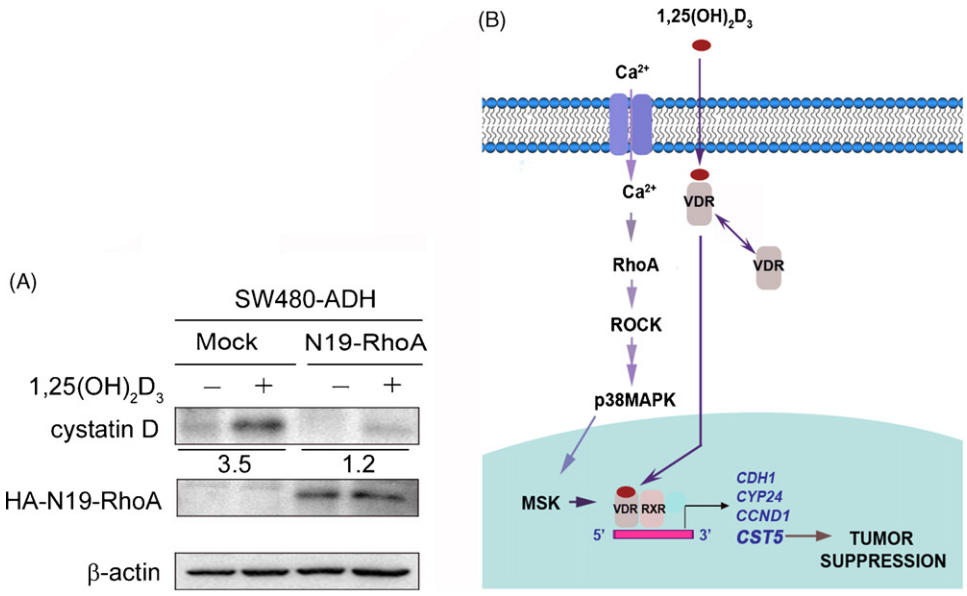

Fig. 4. RhoA is required for *CST5*/cystatin D induction by $1,25(OH)_2D_3$. (A) Lysates of mock and N19-RhoA SW480-ADH cells treated with $1,25(OH)_2D_3$ (10^{-7} M) or vehicle for 48 h, were analyzed by Western blotting with antibodies against cystatin D or HA. β-actin was used as a loading control. (B) Schematic representation of the mechanism of action of $1,25(OH)_2D_3$ in human colon cancer cells. $1,25(OH)_2D_3$ activates RhoA-ROCK and then p38MAPK-MSK following an initial increase in cytosolic Ca^{2+} concentration. This signaling pathway is required for the regulation of target genes such as *CDH1*/E-cadherin, *CCND1*/cyclin D1 or *CST5*/cystatin D which encode proteins with tumor suppressor activity.

tant due to its tumor suppressor activity. *CST5*/cystatin D display wide effects on gene expression and on both cell proliferation and phenotype that were unexpected from a mere inhibitor of cysteine proteases, and that suggest additional activities perhaps in the direct or indirect control of key transcription factors or regulatory proteins. Here we show that the induction of *CST5*/cystatin D by $1,25(OH)_2D_3$ depends also on RhoA activity, which emphasizes the relevance of the RhoA-ROCK and p38MAPK-MSK cascade for $1,25(OH)_2D_3$ action at least in this system. Interestingly, transient RhoA activation has been shown to be crucial for other differentiation processes: in myoblasts it regulates M-cadherin expression and cell fusion linked to terminal differentiation, whereas its sustained activation blocks this process [17]. By contrast, RhoA has a pro-metastatic role in breast and other types of human cancer cells [18]. This apparent contradictory behavior is possibly related to the level and duration of RhoA activation and to its combination with specific genetic and epigenetic alterations.

In summary, our results support a model for vitamin D action in colon cancer cells, and possibly in other systems [9], based on the convergence of a signaling cascade triggered at the plasma membrane by Ca^{2+} influx (and later by cytosolic activation of RhoA-ROCK and p38MAPK) with the modulation within the nucleus of the transcriptional activity of VDR-RXR complexes by MSK (activated either directly or indirectly by p38MAPK phosphorylation; Fig. 4B). Data shown here add *OPN*/osteopontin, *OCN*/osteocalcin and *CST5*/cystatin D to the list of genes whose induction by $1,25(OH)_2D_3$ requires RhoA activation.

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