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Proteomic analysis of 1α,25-Dihydroxyvitamin D_3 action on human colon cancer cells reveals a link to splicing regulation

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ABSTRACT

1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and other vitamin D compounds are promising molecules in the prevention and therapy of colon cancer and other neoplasias. To study the mechanism of action of $1,25(OH)_2D_3$ in colon cancer cells, we carried out a comparative proteomic analysis of the nuclear fractions of SW480-ADH cells treated with $1,25(OH)_2D_3$ or vehicle during 8 or 48 h. 2D-DIGE analysis combined with MALDI-TOF-TOF mass spectrometry interrogation led to the identification of 59 differentially expressed unique proteins. Most identified proteins were nuclear, but several cytoskeleton-associated proteins were also detected. A good concordance between changes in expression at protein and RNA levels was observed for the validated proteins. A large group of identified proteins, such as SFPQ, SMARCE, KHSRP, TARDBP and PARP1, were involved in RNA processing or modification and have been ascribed to the spliceosome compartment of human cells. In addition, a smaller group of proteins (ERM (Ezrin, Radixin, Moesin) family, VCL, CORO1C, ACTB) were cytoskeleton-associated and played a role in cell adhesion and morphology. These results confirm the induction of epithelial phenotype by $1,25(OH)_2D_3$ and suggest a role for vitamin D compounds in the regulation of the spliceosome and thus, in alternative splicing and possibly microRNA synthesis in colon cancer cells.

1. Introduction

Many epidemiological studies have shown an inverse relation between exposure to sunlight, vitamin D_3 (cholecalciferol) intake or 25-hidroxyvitamin D_3 (calcidiol) blood levels and the incidence of several neoplasias, particularly colorectal cancer (CRC) [1–4]. Preclinical data obtained in cultured cells and experimental animals support a protective effect of vitamin D compounds (vitamin D_3, its most active metabolite 1α,25-dihydroxyvitamin D_3 (calcitriol), or less calcemic derivatives) against a variety of cancers [5–7]. Clinical trials have been scarce and inconclusive [7–9], and several are in progress using different vitamin D compounds alone or in combination in patients with different neoplasms (see *ClinicalTrials.gov*).

1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) has anti-proliferative, pro-apoptotic and pro-differentiation effects on many

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tumor cell lines. In human SW480-ADH colon cancer cells that express vitamin D receptor (VDR), $1,25(OH)_2D_3$ induces *CDH1*/E-cadherin expression, an epithelial differentiated adhesive phenotype and antagonizes the Wnt/β-catenin signaling pathway [10]. A global transcriptomic study showed that $1,25(OH)_2D_3$ changes significantly the RNA expression profile of these cells: about two-thirds of the target RNAs were upregulated and one-third were downregulated [11]. These RNAs encoded for proteins involved in many different cell functions including transcription, cell adhesion, DNA synthesis, apoptosis, redox status and intracellular signaling. In-depth functional studies of some of the reported target genes such as *DICKKOPF-1*, *CST5/Cystatin D* or *SPROUTY-2* confirmed their role mediating the protective effect of $1,25(OH)_2D_3$ against colon cancer [12–14].

Several transcriptomic studies searching for RNAs regulated by $1,25(OH)_2D_3$ have been done in different cell types [11,15–17]. However, studies at the protein level in human cancer cells are lacking. In order to get a more comprehensive understanding of the molecular mechanisms of action of $1,25(OH)_2D_3$ we have used 2D-DIGE electrophoresis combined with MALDI-TOF-TOF mass spectrometry to identify proteins regulated by $1,25(OH)_2D_3$ in SW480-ADH cells. We used nuclear extracts from $1,25(OH)_2D_3$- and vehicle-treated cells based on the results of previous transcriptomic studies, which showed that $1,25(OH)_2D_3$ regulates many transcription factors and other nuclear proteins [11]. Moreover, the wide gene regulatory effects of $1,25(OH)_2D_3$ cannot be exclusively mediated by VDR binding to their promoters and should require additional changes in transcription regulators. Finally, the pre-fractionation of the cellular content should facilitate the identification of non-abundant proteins by decreasing those of preferential cytosolic and membrane location. Here, a number of novel $1,25(OH)_2D_3$-regulated proteins were identified. Validation of candidate target proteins was performed by Western blot, immunofluorescence and quantitative PCR analyses. Interestingly, several $1,25(OH)_2D_3$-regulated proteins were related with the spliceosome compartment and RNA metabolism, suggesting a role of $1,25(OH)_2D_3$ on alternative splicing, a process frequently altered in cancer cells [18,19].

2. Materials and methods

2.1. Cell culture

SW480-ADH human colon cancer cells were grown in DMEM supplemented with 10% FCS (Invitrogen). The active vitamin D metabolite $1,25(OH)_2D_3$ was used for cell treatment. Briefly, cells were washed twice with PBS and incubated with 100 nM $1,25(OH)_2D_3$ (with isopropanol as vehicle) in DMEM supplemented with liposoluble hormone-depleted FCS for the indicated times.

2.2. Protein extraction and subcellular fractioning

To obtain nuclear extracts, cell monolayers were washed in PBS and lysed for 15 min in hypotonic buffer (10 mM HEPES pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) supplemented with 1 mM DDT, 1 mM ortovanadate, 1 mM PMSF, 10 mg/ml leupeptin and 10 mg/ml aprotinin. Just before centrifugation (13,000 rpm at 4 °C for 1 min), 10% NP40 was added to the tubes. Supernatants (cytosolic extracts) were conserved at −80 °C until analysis. Pellets containing nuclei were lysed by incubation for 30 min in hypertonic buffer (20 mM HEPES pH 7.5, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA) supplemented as above, and then centrifuged at 13,000 rpm for 10 min at 4 °C. Supernatants (nuclear extracts) were conserved at −80 °C until analysis. Protein expression of cytosolic (β-Tubulin and Rho-GDI) and nuclear (HDAC, Lamin B and VDR) markers was assessed by Western blot to confirm a correct fractioning (Fig. 1A).

2.3. 2D-DIGE electrophoresis

Nuclear extracts were precipitated using methanol/chloroform [20]. Briefly, samples were incubated with 0.1% deoxycolic acid at 4 °C for 10 min. Just before centrifugation (13,000 rpm at 4 °C for 30 min), cold methanol and chloroform were added to the sample tubes. Protein-interphase was washed and centrifuged twice with cold methanol. Pellets were dried and resuspended in 2D lysis buffer (7 M urea, 2 M thiourea, 30 mM Tris–HCl pH 8.5, 4% CHAPS). 2D-DIGE experiments were carried out as previously described [21–23]. Briefly, after protein quantification with RC DC Protein Assay (Bio-Rad), 50 μg of each nuclear protein extract was DIGE labeled (CyDye DIGE Fluor, minimal labeling kit, GE Healthcare) with 400 pmol CyDyes on ice for 30 min and then blocked with L-Lysine for 10 min (incubations were done in the dark on ice). Nuclear protein extracts from SW480-ADH control and $1,25(OH)_2D_3$-treated cells were labeled with Cy3 and Cy5 fluorochromes, respectively. A pool containing equal amounts of extract from each cell condition was labeled with Cy2 dye and was included in all gel runs. First dimension was run on IPG strips (pH 3–10 NL, 18 cm length) (GE Healthcare) in a Multiprotean IEF cell (Bio-Rad) with a 7 steps program (50 V for 10 h, 100 V for 1 h, 250 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 1 h and 8000 V until voltage reached 50 kVh). Second dimension was run overnight on 11% SDS-PAGE in a cooled Protean Plus Dodeca (Bio-Rad). Samples were run for 1 h more after the dye-front run out of the gel. Protein expression patterns were visualized with a Typhoon 9400 scanner (GE Healthcare). Images were processed using two different software for analysis: Redfin Analysis Center-Pro (Ludesi) and Progenesis Samespots (NonLynear Dynamics). Cy3:Cy2 and Cy5:Cy2 ratios for each individual protein were used for quantification. After quantification and normalization of spots, statistical analysis was applied to find significant differences between groups of samples. The 12 spot maps corresponding to 4 replica gels/treatment were used to calculate average abundance changes and paired ANOVA *p*-values and fold-change for each protein across the different gels. Protein spots that showed a significant change in abundance between control and treatment (ANOVA-test $p<0.05$; fold-change >1.25 or <0.80) were selected for further characterization by mass spectrometry.

2.4. Identification of proteins by MALDI-TOF-TOF peptide mass fingerprinting

A preparative gel containing 500 μg of each nuclear extract was run and stained with the Colloidal Blue Staining Kit (Invitrogen) for protein visualization and spot picking after matching against