MicroRNA-22 is induced by vitamin D and contributes to its antiproliferative, antimigratory and gene regulatory effects in colon cancer cells

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Vitamin D deficiency is associated with the high risk of colon cancer and a variety of other diseases. The active vitamin D metabolite 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) regulates gene transcription via its nuclear receptor (VDR), and posttranscriptional regulatory mechanisms of gene expression have also been proposed. We have identified microRNA-22 (miR-22) and several other miRNA species as 1,25(OH)₂D₃ targets in human colon cancer cells. Remarkably, miR-22 is induced by 1,25(OH)₂D₃ in a time-, dose- and VDR-dependent manner. In SW480-ADH and HCT116 cells, miR-22 loss-of-function by transfection of a miR-22 inhibitor suppresses the antiproliferative effect of 1,25(OH)₂D₃. Additionally, miR-22 inhibition increases cell migration per se and decreases the antimigratory effect of 1,25(OH)₂D₃ in both cell types. In silico analysis shows a significant overlap between genes suppressed by 1,25(OH)₂D₃ and miR-22 putative target genes. Consistently, miR-22 inhibition abrogates the 1,25(OH)₂D₃-mediated suppression of NELL2, OGN, HNRPH1, RERE and NFAT5 genes. In 39 out of 50 (78%) human colon cancer patients, miR-22 expression was found lower in the tumour than in the matched normal tissue and correlated directly with that of VDR. Our results indicate that miR-22 is induced by 1,25(OH)₂D₃ in human colon cancer cells and it may contribute to its antitumour action against this neoplasia.

INTRODUCTION

Vitamin D deficiency is increasingly associated with a variety of human diseases (1,2). Among them, colorectal cancer is particularly important as many epidemiological studies link the high risk of developing this neoplasia to low vitamin D diet or circulating level of calcidiol (25-hydroxyvitamin D₃) (3,4). In line with this, experimental data in cultured cells and animal models show that the most active vitamin D metabolite 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) exerts potent protective effects against colon cancer (and other neoplasias) (5–7).

1,25(OH)₂D₃ is a pleiotropic hormone that regulates many genes in numerous tissues in the organism. Its classical model of action implies the binding to, and activation of a member of the superfamily of nuclear receptors, the vitamin D receptor (VDR). VDR acts as a ligand-modulated transcription factor that binds to specific sequences (vitamin D response elements) in target genes and increases or decreases their transcription rate through the interaction with a vast array of
co-activators, co-repressors and chromatin modifier enzymes and remodelling complexes (8,9). Recent data, however, indicate that a number of 1,25(OH)2D3 target genes are regulated by posttranscriptional and/or posttranslational mechanisms (10–12).

MicroRNAs (miRNAs) are short non-coding RNAs with wide gene regulatory activity at the posttranscriptional level. MiRNAs associate with several proteins in RNA silencing complexes that cause mRNA degradation or translation inhibition, or both processes (13). In recent years, miRNAs have been shown to play key roles in cancer as they control the expression of crucial oncogenes and tumour suppressor genes and, accordingly, several miRNAs are either over-expressed or silenced affecting tumour progression and metastasis (14,15).

To gain insight into the action of 1,25(OH)2D3 in colon cancer, we have searched for novel targets by screening with miRNAs microarrays. Among the candidate targets identified, we focused on miR-22 based on previous data, suggesting its tumour suppressor activity (16–21). 1,25(OH)2D3 modulates cell proliferation: it usually has a mild to medium cell-type-dependent inhibitory effect, although stimulatory effects have also been reported (22,23). Our results show that miR-22 is induced by 1,25(OH)2D3 and contributes to its inhibitory effects on the proliferation and migration of colon cancer cells. Moreover, we found that anti-miR-22 expression abrogates the regulation by 1,25(OH)2D3 of the RNA levels of several target genes. Importantly, miR-22 is downregulated in a high proportion of colon tumours and its expression correlates directly with that of VDR. Together, these data show that miR-22 is a target of 1,25(OH)2D3 and mediates in part its protective action against colon cancer.

RESULTS

miR-22 is induced by 1,25(OH)2D3

To study whether 1,25(OH)2D3 affects miRNA expression in human colon cancer, we profiled with miRNA microarrays RNA samples extracted from SW480-ADH cells that were treated for different time points either with 10−7 M 1,25(OH)2D3 or the corresponding amount of vehicle (control). A series of miRNA species were consistently found to be upregulated (i.e. miR-146a, miR-22, miR-222) or downregulated (i.e. miR-203) (Fig. 1). Data have been deposited in GEO databases (GSE34564). On the basis of its kinetics of induction and the literature reporting its tumour suppressive activity in several systems, miR-22 was chosen for an in-depth study.

Validation of microarray data was performed by quantitative real-time PCR (qRT-PCR). The level of miR-22 increased in a time- and dose-dependent manner following 1,25(OH)2D3 treatment of SW480-ADH cells (Fig. 2A and B). Moreover, miR-22 was also induced by this hormone in five others human colon cancer cell lines (LS174T, HT29, SW1417, DLD-1, HCT116), while no induction was found in SW480-R and SW620 cells that lack VDR expression (5) (Fig. 2C).

miR-22 mediates the antiproliferative and antimigratory effects of 1,25(OH)2D3

Next, we examined whether the induction of miR-22 could be relevant for the inhibitory effect of 1,25(OH)2D3 on cell proliferation and migration of colon cancer cells. To this end, we first transfected SW480-ADH and HCT116 cells with a miR-22 oligonucleotide inhibitor (anti-miR-22) or a non-silencing control (scrambled oligonucleotide, SCR) and analysed their proliferation in the presence or absence of 1,25(OH)2D3. In both cell types, the decrease in the number of viable cells, resulting from the sum of effects on cell division and survival caused by the hormone, was blunted by the addition of anti-miR-22 but not by that of SCR oligonucleotide (Fig. 3A and B). Notably, in the absence of 1,25(OH)2D3, anti-miR-22 treatment did not alter cell division (Fig. 3A and B) but, in contrast, led to an increased migratory capacity in transwell assays (2.13 ± 0.3-fold, P = 0.0002, for HCT116 cells; 1.75 ± 0.38-fold, P = 0.007, for SW480-ADH cells) (Fig. 4A and B). In line with this, anti-miR-22 abolished the inhibition of cell migration caused by 1,25(OH)2D3 in both cell types (Fig. 4A and B). In all experiments, blockade of miR-22 induction by 1,25(OH)2D3 using anti-miR-22 was analysed by qRT-PCR (Supplementary Material, Fig. S1).

miR-22 mediates the regulation of several 1,25(OH)2D3 target genes

We wished to explore the putative role of miR-22 in the gene regulatory effect of 1,25(OH)2D3. To this end, we first did a comparative in silico analysis by using TargetScan (24) for predicted miR-22 targets and data from our transcriptomic studies of 1,25(OH)2D3 target genes (25) and unpublished data. This study rendered that 9 out of 36 genes (25%) downregulated and 11 out of 93 genes (11.8%) upregulated by the
hormone in SW480-ADH cells are putative miR-22 targets (2.11-fold enrichment down- versus up-regulation) (Fig. 5A and Supplementary Material, Table S1). The comparison of these data with the predicted targets of a randomly selected group of miRs (miR-200a, miR-142-3p, miR-142-5p, miR-320a, miR-31, miR-365 and miR-34b; median of total targets of 655 and median overlap with 1,25(OH)2D3 targets of only 3) revealed the statistical evidence that miR-22 targets are enriched in the 1,25(OH)2D3-treated array data set relative to other miRs.

To validate this finding, we analysed the expression of a few selected genes downregulated by 1,25(OH)2D3, such as neural tissue-specific epidermal growth factor-like repeat domain-containing protein (NELL2), osteoglycin (OGN), heterogeneous nuclear ribonucleoprotein H1 (HNRPH1), nuclear factor of activated T cells 5 (NFAT5), caudal type homeobox 2 (CDX2) and arginine-glutamic acid dipeptide (RE) repeats (RERE) in SW480-ADH and HCT116 cells transfected with either anti-miR-22 or SCR oligonucleotides. Supporting a role of miR-22 mediating the downregulation of these genes by the hormone, in either or both cell lines, the transfection of anti-miR-22 oligonucleotides but not of SCR abrogated such inhibitory effect, except in the case of CDX2 (Fig. 5B and C). We also studied CDH1, a gene transcriptionally upregulated by the hormone that mediates part of its effects in colon cancer cells (5,26). As expected from this regulation, anti-miR-22 did not affect the induction by 1,25(OH)2D3, suggesting that CDH1 mRNA may not be indeed a target of miR-22.

Expression of miR-22 in human colon tumours

Finally, we studied the expression of miR-22 in 50 matched normal and tumour samples from human colon cancer patients. In agreement with a tumour suppressive action, miR-22 expression was found downregulated in 39 out of 50 (78%) tumours when compared with normal tissue.
In line with previous studies (27–29), VDR expression was downregulated in 36 out of 50 (72%) tumours versus normal tissues (Supplementary Material, Fig. S2). Notably, a significant direct correlation was found between the expression of miR-22 and VDR RNA (Spearman correlation coefficient, $r = 0.315$, $P = 0.026$) (Fig. 6B), which suggests that the VDR-mediated induction of miR-22 observed in cultured cells probably also takes place in human colon tissue.

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Figure 5. Expression of anti-miR-22 abolishes the downregulation of several genes by 1,25(OH)₂D₃. (A) Venn diagram representing the overlap between miR-22-predicted targets (TargetScan) and 1,25(OH)₂D₃-modulated genes identified in microarray analyses of SW480-ADH cells. qRT-PCR analysis of NELL2, OGN, HNRPH, REE, CDX2, CDH1 and NFAT5 mRNA expression in HCT116 (B) or SW480-ADH (C) cells treated for 48 h with 10⁻⁷ M 1,25(OH)₂D₃ or vehicle. SDHA was used for normalization.
DISCUSSION

In this study, we identify miR-22 as a target of 1,25(OH)_{2}D_{3} in human colon cancer cells that mediate in part its inhibitory effect on cell proliferation and migration. The finding that an anti-miR-22 reduces the antiproliferative effect of 1,25(OH)_{2}D_{3} strongly supports that the induction of miR-22 contributes to, and is not a mere consequence of the growth inhibitory action of the hormone. Moreover, miR-22 is found to mediate the repression by 1,25(OH)_{2}D_{3} of several genes, such as OGN, NELL2, HNRPH1, RERE and NFAT5 at the RNA level, which we have validated as targets of this hormone in human colon cancer cells.

Supporting the consistency of these findings, they have been described in two cell lines that harbour different sets of mutations that are crucial and represent most human colon tumours: while SW480-ADH cells harbour mutated APC, TP53 and K-RAS genes, HCT116 cells express a wild-type APC but a mutated CTNNB1/β-catenin that is the alternative responsible mechanism for the aberrant activation of the Wnt canonical
signalling pathway, a hallmark of this neoplasia. Both cell types contain a mutated K-RAS, but they differ with respect to the major tumour suppressor TP53, which is normal in HCT116 but mutated in SW480-ADH cells.

Several reasons support a role of the regulation of miR-22 for 1,25(OH)_{2}D_{3} action in this system. First, our data show that miR-22 mediates the antiproliferative and antimigratory action of the hormone. Secondly, because the repression by 1,25(OH)_{2}D_{3} of certain genes that are in part dependent on miR-22 may contribute to its antitumoural action; thus, NELL2 is repressed by the antitumour agent genistein in pancreatic cancer Panc1 cells (30), is over-expressed in Burkitt’s lymphoma cells, neuronal tumours and benign prostatic hyperplasia (31–33) and contributes to the survival-promoting effects of estradiol via the extracellular signal-regulated kinase signalling pathway (34). HNRPH1 encodes a splicing regulator that is overexpressed in colon cancer and counteracts apoptosis induced by etoposide and fluoropyrimidine anticancer drugs (35–37). NEAT1 transcriptional activity is induced by integrin α6β4 and Src oncogene (38), and mediates carcinoma invasion through the induction of S100A4 (39), and possibly also melanoma invasion (40). RERE (or ATN1) encodes a nuclear receptor corepressor that is aberrantly expressed in neuroblastoma and appears to be involved also in acute myeloid leukaemia (41). Very little relation exists between OGN and cancer; paradoxically, it has been proposed to decrease gelatinase activity of murine hepatocarcinoma cells (42).

The relevance of miR-22 regulation by 1,25(OH)_{2}D_{3} is also supported by the tumour suppressive effects of this miRNA recently described in other systems. Thus, miR-22 suppresses cell proliferation and tumourigenicity and is downregulated in hepatocellular carcinoma (16), represses c-Myc-binding protein, MYCBP, a positive regulator of the strong oncogene c-MYC (17), and controls the EVI-1 oncogene in breast cancer cells (18). Additionally, miR-22 is induced by p53 and favours p53-dependent apoptosis by targeting CDKN1A/p21^{cip1} (19), although our data show that the induction of miR-22 by 1,25(OH)_{2}D_{3} is independent of p53 as it takes place in cells with either wild-type or mutant TP53 gene. Also, miR-22 suppresses the activity of nuclear factor kappa B, an important inducer of cell survival and inflammatory and tumourigenic cytokines (20). Remarkably, miR-22 may have an anti-angiogenic effect in colon cancer via the inhibition of hypoxia inducible factor-1α expression (21). Lastly, it has recently been reported the additive induction of miR-22 by testosterone and 1,25(OH)_{2}D_{3} in the prostate cancer LNCaP cell line (43). Altogether, these data suggest that miR-22 induction may play a role in the antitumoural action of 1,25(OH)_{2}D_{3}.

Data obtained from human biopsies show the correlation between the expression of VDR RNA and miR-22, suggesting that 1,25(OH)_{2}D_{3} may also regulate miR-22 expression in vivo. Likewise, the downregulation of miR-22 concomitant to VDR silencing in tumours agrees with its tumour suppressive effects in cultured colon cancer cells.

In summary, we have identified miR-22 as a novel target of 1,25(OH)_{2}D_{3} that expand the range of its gene expression modulatory activity at the posttranscriptional level and may contribute to explain at least partially its protective action on this important neoplasia.

MATERIALS AND METHODS

Cells and cell culture

Human colon cancer SW480-ADH, HCT116, HT29, LS174T, DLD-1, SW620, SW1417 and SW480-R cell lines were cultured in DMEM plus 10% fetal bovine serum (Invitrogen). All experiments using 1,25(OH)_{2}D_{3} or isopropanol (vehicle) were performed in medium supplemented with charcoal-treated serum.

Cell proliferation and migration assays

To measure proliferation, cells (15 × 10^{3}) were seeded in 24-well plates and treated for up to 3 days with 10^{-7} M of 1,25(OH)_{2}D_{3} or vehicle. Living cells were counted after trypsinization using a TC10 TM Automated Cell Counter (Bio-Rad). For migration assays, cells were transfected with antisense or control oligonucleotides and 12 h later they were trypsinized and counted. Equal numbers (15 × 10^{4}) were seeded on the upper surface of 8.0 μm pore Transwells® (Corning Incorporated). 1,25(OH)_{2}D_{3} (10^{-7} M) or vehicle was added to the upper and lower media. After 24 h incubation, cells on the upper surface of the filter were removed by using a cotton swab and those attached to the lower surface of the filters were stained using Diff-Quick reagents (Dade Behring) and counted (10 fields/Transwell®). Experiments were performed in triplicate. Phase-contrast images were captured with a Leica DC300 digital camera mounted on an inverted Leitz Labovert FS microscope. All images were processed using Adobe Photoshop CS4 software.

miRNA microarray analysis

Microarrays were produced in the Genomics Unit of the Spanish National Cancer Research Centre (CNIO), Spain. Briefly, NCode Multi-Species miRNA V2 probe set (Invitrogen, cat. # MIRPS2-01) was printed on Nextreration epoxy E slides (Schott) by following manufacturer’s recommendations. Probe sequences target all of the known mature miRNAs in the Sanger miRBase Sequence Database, Release 9.0. Cellular small RNA fractions were extracted with PureLink miRNA isolation kit (Invitrogen) and labelled with the 2-color LabelIT miRNA labelling system (Mirus). Extracts from cells treated with 1,25(OH)_{2}D_{3} or vehicle at each time point were compared in dye-swapped hybridizations. Hybridization conditions were as per Mirus’ kit recommendations and microarrays were read with an Agilent G2505B scanner. Two hybridization batches were performed on a first series of cells cultured for 24, 48 or 72 h (data not shown). A last batch, in which all the samples were dye swapped in technical replicates, employed new cultures from a time series of 24, 48 and 96 h. Changes between 1,25(OH)_{2}D_{3} and vehicle treatments were apparent and steady but small, and statistically non-significant. Biological replication (two replicates for time points 24 and 48 h) was insufficient. Entities that showed no signs of differential expression, with absolute fold change < 1.5, were discarded from consideration. Raw data from microarray images were quantified, background subtracted and global Lowess normalized with Feature Extraction Software (Agilent). Visualization of miRNA expression data showing relatively high intensity signals was carried out by
importing processed data in MultiExperiment Viewer v4.7 (44) and MS Excel. The expression data set was filtered to include only those probe sets detecting miRNAs with mean expression values showing at least a change of ± 0.5 (log₂ scale) between each pair of samples under comparison. Validation was carried out by qRT-PCR analysing three independent sets of samples.

Transfection and miR-22 silencing
To silence miR-22, cells were transfected with 25 nM of miRIDIAN anti-miR-22 (hairpin inhibitor oligonucleotide) or with a Caenorhabditis elegans miRNA not found in humans [miRIDIAN miRNA Hairpin Inhibitor Negative Control 1 (SCR)] (Dharmacon) using the jetPEI reagent (PolyPlus Transfection) following manufacturer’s guidelines. Experiments were performed up to 72 h after transfection and the level of miR-22 silencing was monitored by qRT-PCR.

Quantitative RT-PCR
Total RNA (including small RNAs) from cultured cell lines was extracted using the NucleoSpin® miRNA extraction kit (Macherey-Nagel). RNA from ~30 μg of tumour or normal tissue was extracted using RNeasy mini kit (Qiagen). qRT-PCR analyses of miR-22 expression level were performed using the miRNA-specific TaqMan MicroRNA Assay Kit (Applied Biosystems). Briefly, 12.5 ng of total RNA was reverse-transcribed using the corresponding RT Primer and the TaqMan MicroRNA Reverse transcription Kit (Applied Biosystems). PCR was performed on 1.33 μl of RT products by adding the TaqMan PCR primers and the iQ Supermix (Bio-Rad). RNU44 small RNA was used for normalization of input RNA/cDNA levels. VDR, NFAT5, NELL2, OGN, CDX2, CDH1, RERE and HNRPH1 RNA levels were measured using the primers listed in Supplemental Material, Table S2 and the Power SYBR® Green PCR Master Mix (Applied Biosystems). RNA expression values were normalized versus the housekeeping gene succinate dehydrogenase complex subunit A (SDHA). The reaction was performed in a CFX384 Real-Time PCR Detection System (Bio-Rad).

Patients and tumour samples
Normal and tumour tissue samples from 50 colon cancer patients were obtained immediately after surgery, immersed in RNA later (Applied Biosystems), snap-frozen in liquid nitrogen and stored at −80°C until processing. Tumours were considered sporadic cases because no clinical antecedents of Familial Adenomatous Polyposis were reported and those with clinical criteria of hereditary non-polyposis colorectal cancer (HNPPC) (Amsterdam criteria) were excluded. Tumours were examined by two different pathologists to: (i) confirm adenocarcinoma diagnosis and presence of at least 75% of tumour tissue in the sample, (ii) determine the histological level of the tumour, and (iii) verify the absence of tumour cells in normal tissue. All patients gave written informed consent. The protocol was approved by the Research Ethics Board of the Hospital Universitario Puerta de Hierro, Majadahonda, Madrid, Spain.

Statistical analysis
Results are expressed as mean ± SD unless otherwise specified. Statistical significance was assessed by the one-way analysis of variance test with Bonferroni post-test. Differences were considered significant when P < 0.05. The single asterisk indicates P < 0.05, the double asterisk P < 0.01 and the triple asterisk P < 0.001. All statistical analyses were performed using the Prism software V5 (GraphPad software). As the tumour/normal tissue ratios of VDR and miR-22 expression were not normally distributed (Kolmogorov–Smirnov test, Lilliefors correction), we normalized the data distribution by using log₁₀ for statistical analysis. Correlations between RNA expression levels were analysed using the Spearman correlation coefficient.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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CONFLICT OF INTEREST STATEMENT
None declared.

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