MicroRNA-32 Upregulation by 1,25-Dihydroxyvitamin D₃ in Human Myeloid Leukemia Cells Leads to Bim Targeting and Inhibition of AraC-Induced Apoptosis

Elzbieta Gocek¹,², Xueling Wang¹, Xiuping Liu³, Chang-Gong Liu³, and George P. Studzinski¹

Abstract

1,25-Dihydroxyvitamin D₃ (1,25D) used to treat human acute myeloid leukemia (AML) cells induces features of normal monocytes, but the mechanisms underlying this response are not fully understood. We hypothesized that one or more microRNAs (miRNA) known to control mouse hematopoiesis and lineage commitment might contribute to the ability of 1,25D to control the malignant phenotype. Here we report that 1,25D markedly induces expression of miR-32 in human myeloid leukemia cells, in which it targets the 3'-untranslated region of the mRNA encoding the proapoptotic factor Bim to reduce its expression. RNAi-mediated suppression of the miRNA-processing enzymes Drosha and Dicer increased Bim levels, in support of the concept that Bim is under miRNA control in AML cells. Antisense-mediated suppression of miR-32 was sufficient to upregulate Bim expression in AML cells. Conversely, ectopic expression of miR-32 downregulated Bim expression and increased the differentiation response to 1,25D treatment in a manner that was associated with increased cell survival. The positive effects of miR-32 on cell survival were confirmed by evidence of increased cell death in AML cells preexposed to antisense miR-32 before treatment with arabinocytosine, a chemotherapeutic drug used to treat human AML. Together, our findings indicate that miR-32 blockade is sufficient to elevate Bim expression and sensitize AML cells to chemotherapy-induced apoptosis. Thus, agents which can inhibit miR-32 expression may offer clinical utility by enhancing therapeutic efficacy in human AML. Cancer Res; 71(19); 6230–9. ©2011 AACR.

Introduction

Acute myeloid leukemia (AML) is a hematologic disease characterized by blocks at various stages of hematopoietic differentiation, which lead to uncontrolled cell proliferation and accumulation of immature myeloid cells in bone marrow and the peripheral blood. The disease has extremely poor prognosis, even with the available treatment regimens, which currently are based on the eradication of the malignant stem and myeloid precursor cells (blasts) by cytotoxic agents such as arabinocytosine (AraC). Unfortunately, toxicity of these drugs to the patients limits their dosage, and recurrences of the disease are frequent (1). Thus, effective other treatment modalities are urgently needed.

The differentiation blocks responsible for the disease can be overcome in cultured AML cells by supraphysiologic concentrations of the active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25D; refs. 2–4). The rationale for the ability of 1,25D to achieve this effect has been presented as due to an elevation of the levels of transcription factors (TF), for example, jun/AP1 and C/EBP beta, which permit the blasts to bypass the barrier presented by the leukemia-causing mutations, frequently by a switch from myeloid to monocytic differentiation lineage induced by these TFs (5–9). However, although all-trans retinoic acid (ATRA) has been successfully used in the clinic, induction of differentiation of AML blasts by 1,25D has so far not resulted in notable clinical success. The reasons for this may include the fact that in addition to prodifferentiation activity, ATRA also promotes cell death and is particularly effective when combined with arsenic trioxide, a toxic agent (10, 11). Similarly, increased cytotoxicity is seen when, following AraC exposure, 1,25D is added to cultured AML cells (12, 13). It seems reasonable, therefore, to explore whether changes in cell survival mechanisms that accompany 1,25D-induced differentiation can be modified to increase the therapeutic potential of 1,25D.

Using a miRNA microarray platform, we observed that miR-32 was the most highly elevated miRNA in human leukemia 60 (HL60) cells treated with 1,25D. One of the predicted targets of miR-32 lies in the 3'-untranslated region (UTR) of BCL2L11 gene, which encodes the proapoptotic protein Bim (14, 15). Therefore, we investigated whether the increased levels of miR-32 in 1,25D-treated AML cells can be validated by quantitative real time PCR (qRT-PCR), and if so, whether miR-32 regulates the expression of Bim, and whether this is

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associated with changes in the ability of cytotoxic agents, such as AraC, to induce the death of AML cells.

Materials and Methods

Cell culture
HL60-G cells, derived from a patient with promyeloblastic leukemia (16), and U937 cells, obtained from American Type Culture Collection were cultured in suspension in RPMI-1640 medium supplemented with 10% bovine calf serum (HyClone) in a humidified atmosphere containing 5% CO₂ at 37°C. For U937 cells, concentrations of 1.25D routinely used was 10 nmol/L, as these cells are less sensitive to 1.25D than HL60 cells, which were treated with 1 nmol/L 1.25D. Cells were passaged 2 to 3 times a week and were used in the exponential growth phase. Cells were routinely tested for Mycoplasma by selective culture techniques. For all experiments the cells were resuspended in fresh medium, and each experiment was repeated at least 3 times.

Isolation of mononuclear cells from peripheral blood and selection of monocytes
Peripheral blood samples were obtained from 5 healthy volunteers according to Institutional Review Board protocol. Mononuclear cells were isolated by using Histopaque-1077 (Sigma-Aldrich), as previously described (17). Monocytes were positively selected with CD14 MicroBeads (Miltenyi Biotec Inc.) as directed by manufacturer’s protocol. Homogeneity of CD14-positive cells was determined by using EPICS XL flow cytometer (Beckman Coulter).

Chemicals and antibodies
1.25D was a kind gift from Dr. Milan Uskokovic (Bioxell). Antisense oligonucleotides against Drosha (Cat#L-016996-00) and Dicer (Cat#L-003483-00) were obtained from Thermo Scientific, hsa-miR-32 anti-miR miRNA inhibitor (ID: AM12716) and hsa-miR-32 pre-miR miRNA precursor (ID: PM12716) were obtained from Applied Biosystems. Complete protease inhibitor cocktail was purchased from Hoffmann-La Roche. Antibodies were procured from Cell Signaling Technologies [Bim, Bax, secondary anti-rabbit, and anti-mouse linked to horseradish peroxidase (HRP)] and from Santa Cruz Biotechnology (Crk-L and Calregulin).

MicroRNA target predictions and pathway analysis
Public web-based prediction sites under miRbase were used to identify miRNA 32 target binding sites in the 3’-UTR of human gene transcripts (18). miRBase currently links miRNA-32 to targets predicted by micromirs Targets (19), microRNA.org (20), TargetScan (21), and Pictar-Vert (22), and aims to provide a more extensive target prediction aggregation service in the future. In addition, other target prediction online softwares (DIANA-microT, miRanda, and PITA) were used. Targets of miRNAs which were differentially and significantly (>1.5-fold change and P < 0.05) expressed by 1.25D were subjected to Ingenuity Pathway Analysis (IPA: Ingenuity Systems) done by uploading specific miRNA lists into miRNA array analysis program. The list of gene targets of miRNAs predicted by IPA was filtered to remove duplicates and genes with no annotation in IPA listed apoptosis pathways, resulting in a list of network-eligible genes associated with the apoptosis signaling pathway.

Transfection of siRNA against Drosha and Dicer, of antisense oligonucleotides against miR-32, and of miR-32 precursor
This was carried out using Endo-Porter delivery reagent from Gene Tools Inc. Si-Drosha, si-Dicer (Thermo Scientific), anti-miR-32 inhibitor (a chemically modified, single-stranded nucleic acid designed to specifically bind to and inhibit endogenous miRNA molecules), pre-miR-32 (Ambion) and appropriate nontargeting control oligonucleotides were transfected at a final concentration of 20 nmol/L for 48 hours before exposure to other compounds.

RNA isolation and qRT-PCR
Total RNA was extracted by using Trizol (Invitrogen) according to manufacturer’s protocol and reverse transcribed for quantification by TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) as previously described (23). Mature miRNAs were quantitated using 2-step TaqMan RT-PCR with TaqMan microRNA kit. MiR-32 expression level was normalized using U6 rRNA as an internal control (Applied Biosystems).

qRT-PCR for Bim was carried out using a FastStart DNA SYBR Green PCR kit (Roche Diagnostics) as described before (23). Fold changes of mRNA levels in target gene relative to the RNA polymerase II (RPII) control were calculated by relative quantification analysis. Primers used for Bim were as follows: upstream 5’-AGTTCTGAGTTGACGGAGAGTG-3’, downstream 5’-TCTGTTCGCACTGCTGCTGAC-3’; for RP II, upstream 5’-GCACCACGTTCCAAATGAC-3’, downstream 5’-GTGCCG CTGCTTCCATAA-3’. The quality of PCR products were monitored using post-PCR melting curve analysis.

Markers of monocytic differentiation
Aliquots of 1 × 10⁶ cells were harvested, washed twice with PBS and incubated for 45 minutes at room temperature with 0.5 μL MY4-RD-1 and 0.5 μL M01-FITC (fluorescein isothiocyanate) antibodies (Beckman Coulter Inc.) to analyze the expression of monocytic differentiation surface markers CD14 and CD11b, respectively. The cells were then washed 3 times with ice-cold PBS, resuspended in 1 mL PBS, and analyzed using EPICS XL flow cytometer (Beckman Coulter). Isotypic mouse IgG1 was used to set threshold parameters.

Cell-cycle distribution
The DNA content of the cells was determined as follows: 1 × 10⁶ cells were harvested and washed twice with PBS, then fixed with 75% ethanol at −20°C for 24 hours. Cells were then collected and resuspended in 1 mL of PBS with 10 μg/mL RNase (Sigma) and 10 μg/mL propidium iodide (PI; Sigma) for 30 minutes at 37°C. PI-stained cells were analyzed using EPICS XL flow cytometer. The resultant distribution of DNA content was gated and analyzed using the multicycle program to determine the proportion of cells in the sub-G₁/G₀ fraction, which represents the nonviable cells.
Annexin V and propidium iodide staining

AML cells were induced to apoptosis by 100 µmol/L AraC (Sigma) for 24 hours. Samples were collected, washed once with PBS, and resuspended in the 10 mmol/L HEPES/NaOH binding buffer, containing 0.14 mol/L NaCl and 2.5 mmol/L CaCl₂, pH 7.5. Apoptotic cells were stained using an Annexin V binding buffer, containing 0.14 mol/L NaCl and 2.5 mmol/L CaCl₂, pH 7.5. Annexin V and PI positive/C6 percentagess of the cell population (mean ± SE). Significance of the differences from the corresponding controls, P < 0.05; bars represent mean values ± SE, n = 8–12 for cell lines, n = 5 for monocytes. CTL, control; MI, median intensity of signal; FC, fold change from CTL; GM-CSF, granulocyte-macrophage colony stimulating factor (20 ng/mL), used to support survival/growth of monocytes in primary culture.

Western blotting

Western blotting was done using whole-cell extracts as described before (23). Briefly, membranes were incubated overnight with different primary antibodies and then blotted with a HRP-linked secondary antibodies for 1 hour. The protein bands were visualized using a chemiluminescence assay system (Pierce Biotechnology), each membrane was stripped and reprobed for internal control (Crk-L or calregulin). The optical density of each band was quantitated using ImageQuant 5.0 (Molecular Dynamics).

Statistical methods

Each experiment was conducted at least 3 times. The results were expressed relative to vehicle controls, or as percentages of the cell population (mean ± SE). Significance of the differences between mean values was assessed by a 2-tailed Student’s t test. All computations were done with an IBM-compatible personal computer using Microsoft EXCEL.

Results

Upregulation of miR-32 by 1,25D in HL60 and U937 cells

The initial experiments were conducted using a miRNA microarray platform (24) to identify the expression of miRNAs after exposure to 1,25D. IPA indicated that only miR-32 was differentially expressed in both HL60 and U937 1,25D-treated cells. As shown in Figure 1A, miR-32 was markedly (~11-fold) and highly significantly increased in HL60 cells, but the increase was less marked and below the level of statistical significance in U937 cells. However, validation of these results by quantitative real-time PCR, shown in Figure 1B, showed that although the increase in miR-32 level was less marked in U937 than in HL60 cells, the increase was statistically significant in both cell lines. Although TaqMan results were less dramatic than those obtained by the microarray, they were clearly confirmatory.

Treatment with 1,25D of monocytes isolated from healthy volunteers also significantly (P < 0.01) increased miR-32 levels.

The proapoptotic protein Bim is a potential target of miR-32 in AML cells

In silico studies reported in at least 6 public searches (Fig. 2A) revealed that 3’-UTR of BCL2L11 gene, which encodes the proapoptotic protein Bim (14, 15), has a potential miR-32 binding site at chromosome 2q13, which is conserved between human and murine genomes (Fig. 2B). Direct regulation in AML cells of Bim protein expression by miR-32 was suggested by the marked decrease in Bim mRNA and Bim protein, its largest isoform known as Bim-EL (15), in both HL60 and U937 cells after exposure to 1,25D (Fig. 2C). This is consistent with the report that in human prostate cancer cells LNCaP, a luciferase reporter construct containing BCL2L11 3’-UTR with...
the predicted miR-32 target sequence, showed translational inhibition by miR-32 (25). Although cell type–restricted specificity of miRNA targets is well known, this suggests that Bim may be a general target of miR-32 in human cells.

Expression of Bim is regulated by miRNAs

To confirm that the above predictions apply to AML cells, we first tested whether an interference with miRNA processing affects Bim protein expression in these cells. To accomplish this we reduced the levels of the RNases which process miRNA precursors, the RNase III Drosha, which processes miRNAs in the nucleus, and the cytosolic RNase III, Dicer (26–29). As shown in Figure 3A, transfection of silencing constructs of Dicer and Drosha effectively abrogated 1,25D-induced upregulation of miR-32 expression, though the ambient levels of miR-32 transcripts were not sufficiently reduced to detect statistical significance. As expected, the changes in Bim protein levels were reciprocally affected by the reduction in miRNA-processing enzymes, with decreased Bim levels noted after 1,25D exposure, which were abrogated and actually increased by Dicer and Drosha (Fig. 3B). The increase in Bim levels when miRNA processing is reduced corroborates that Bim expression is regulated by a miRNA or several miRNAs.

Precursor and antisense miR-32 regulate Bim protein levels

To establish that miR-32 is at least one of the miRNAs which affected Bim protein expression when the processing of
miRNAs was inhibited, we used antisense miR-32 oligonucleotides (anti–miR-32) and precursor miR-32 (pre–miR-32) to modulate cellular levels of miR-32. Under the conditions employed, anti–miR-32 produced a significant decrease in the cellular levels of endogenous miR-32 (Fig. 4A, bars 3–4) with corresponding increases in Bim mRNA (Fig. 4B, bars 3–4) and protein (Fig. 4C). Conversely, pre–miR-32 resulted in significant increases in cellular levels of miR-32 (Fig. 4A, bars 7–8), whereas Bim mRNA (Fig. 4B, bars 7–8) and protein (Fig. 4D) levels decreased. This indicates that Bim is a target of miR-32 in AML cells, and that 1,25D-induced downregulation of Bim can be reversed by anti–miR-32. In contrast, the levels of Bax, another proapoptotic protein, are essentially unaltered by the manipulation of miR-32 cellular levels (Fig. 4C and D).
**MicroRNA-32 Regulates Bim and Promotes Cell Survival**

**Precursor miR-32 promotes 1,25D-induced AML cell differentiation, whereas antisense miR-32 inhibits differentiation**

It has been observed that the processes associated with cell maturation and acquisition of function include cell increased cell survival capacity (30–32). We therefore determined the effect on 1,25D-induced differentiation of the modulation of cellular miR-32 levels by anti–miR-32 or pre–miR-32 and found that anti–miR-32 inhibited cell differentiation (Fig. 5A), whereas pre–miR-32 enhanced differentiation (Fig. 5B). Thus, the changes in the expression of cell surface markers of the monocytic phenotype CD11b and CD14 (Fig. 5) paralleled the cellular levels of miR-32 shown in Figure 4A. The enhancement of monocytic differentiation by pre–miR-32 was also shown by the increased expression of the cytoplasmic esterase NSE, another marker of monocytic phenotype (data not shown). Thus, perhaps indirectly, miR-32 has a minor but clear role in monocytic differentiation.

**Inhibition of miR-32 expression by anti–miR-32 increases the toxicity of AraC to AML cells**

The observed effects of miR-32 on the expression of Bim protein suggest that a reduction of cellular levels of miR-32 should make AML cells more susceptible to the therapeutic agents used to treat this disease, generally AraC. This was tested by preincubating AML cells for 48 hours with anti–miR-32, then treating the cells with 100 μmol/L AraC for 24 hours, and determining Trypan Blue permeability, an indication of necrosis, and the estimation of apoptosis by Annexin V, as well as by the sub G1/G0 fraction obtained from flow cytometric measurement of cell-cycle distribution. All 3 sets of determinations showed that anti–miR-32 increases cell death induced...
by AraC (Table 1, groups 3 and 4, 7, and 8) and abrogates the previously reported antiapoptotic effect of 1,25D (31). The protective effect of 1,25D is seen here for HL60 cells (Table 1, group 1 vs. group 2), although the complexity of the transfection system does not make this apparent in U937 cells. Anti–miR-32 also enhanced apoptosis when doxorubicin or daunomycin were used as the toxic agents (data not shown).

The complementary approach, the use of pre–miR-32, to show the protective effect of miR-32 on AML cell survival, confirmed the protective effect (Table 1). In the experiments in which pre–miR-32 was transfected the changes in cell survival were less marked than those obtained with anti–miR-32, with AraC-induced necrosis not being significantly affected, and only early apoptosis detectable in U937 cells not treated with 1,25D (Table 1, group 15). This is perhaps seen because other components of cell survival network play a greater role than Bim when pre–miR-32 reduces Bim levels, although increased Bim levels in cells treated with anti–miR-32 have a more dominant effect on cell survival. Taken together, the 2 approaches clearly show that the response of AML cells to therapeutic cytotoxic agents may be increased by lowering cellular miR-32 levels. This raises the possibility that the combination of differentiation (by 1,25D) with cytotoxic (AraC) therapy may be further enhanced by agents or conditions which lower cellular miR-32 levels, such as anti–miR-32.

**Discussion**

The studies reported here provide several mechanistic insights and may also have a translational significance. They reveal a novel aspect of the mechanism of vitamin D action, have a bearing on the specificity of miRNA targets, and enhance the understanding of AML cell survival mechanisms. The latter may point the way to increasing the effectiveness of AraC-based induction of disease-free patient survival.
MicroRNA-32 Regulates Bim and Promotes Cell Survival

Table 1. Toxicity of AraC to AML cells is modulated by miR-32

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cells</th>
<th>Treatment</th>
<th>Viability Mean±SD (%)</th>
<th>Early-Apts Mean±SD (%)</th>
<th>Late-Apts Mean±SD (%)</th>
<th>Total-Apts Mean±SD (%)</th>
<th>SubG1 Mean±SD (%)</th>
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<tr>
<td>Anti-miR-32</td>
<td>HL60 Scrambled-A-C</td>
<td>79.3 ± 6.8</td>
<td>9.6 ± 2.9</td>
<td>22.3 ± 6.4</td>
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<td></td>
<td>75.3 ± 1.05</td>
<td>7.6 ± 1.7</td>
<td>16.7 ± 7.7</td>
<td>24.3 ± 6.6</td>
<td>6.0 ± 2.1</td>
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<tr>
<td></td>
<td>65.3 ± 8.3</td>
<td>9.7 ± 1.3</td>
<td>31.9 ± 7.9</td>
<td>41.6 ± 6.5</td>
<td>16.6 ± 4.7</td>
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<tr>
<td></td>
<td>53.0 ± 10.3</td>
<td>10.9 ± 2.4</td>
<td>31.3 ± 7.7</td>
<td>42.2 ± 9.5</td>
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<td>70.3 ± 5.0</td>
<td>10.4 ± 1.2</td>
<td>33.3 ± 6.6</td>
<td>43.7 ± 7.0</td>
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<td>71.7 ± 4.2</td>
<td>9.5 ± 1.7</td>
<td>32.1 ± 3.6</td>
<td>41.6 ± 4.1</td>
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<td>61.0 ± 5.5</td>
<td>10.5 ± 2.1</td>
<td>45.1 ± 6.9</td>
<td>55.6 ± 3.1</td>
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<td>63.3 ± 3.8</td>
<td>9.1 ± 2.4</td>
<td>40.7 ± 7.0</td>
<td>49.8 ± 4.2</td>
<td>12.5 ± 2.7</td>
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<tr>
<td>Pre-miR-32</td>
<td>HL60 Scrambled-P-C</td>
<td>77.0 ± 11.5</td>
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<td>36.3 ± 6.7</td>
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<td>79.0 ± 10.5</td>
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<td>19.9 ± 4.9</td>
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NOTE: Inhibition of miR-32 expression by antisense oligonucleotides increases the toxicity of AraC to HL60 and U937 cells. HL60 and U937 cells were transfected with 20 nmol/L antisense oligonucleotides against miR-32 for 48 hours and then exposed to 1 nmol/L (HL60) or 10 nmol/L (U937) 1,25D for further 48 hours. Then the cells were exposed to 100 μmol/L AraC for further 24 hours. To detect apoptosis, cells were stained using 50 μg/mL Annexin V and 20 μg/mL PI and analyzed by flow cytometry. Annexin V–positive/PI–negative cells were considered as early apoptotic, cells doubly positive, as late apoptotic. Bold type shows the statistically significant differences from the corresponding scrambled controls. P < 0.05; mean values ± SD are shown, n = 4. C, vehicle control; D, 1,25D-treated; A, anti–miR-32; P, pre–miR-32; Apts, apoptosis.

Previous work in our and other laboratories has shown that 1,25D-induced differentiation of human AML cells is accompanied by increased cell survival capacity, which is likely to be multifactorial. These include activation of the ERK, an AKT pathways (33–36), although the specific details about the sequence of molecular events are few. One possibility is that the hKSR2 gene, which is directly upregulated by 1,25D (37), provides an upstream platform for activation of mitogen-activated protein kinase pathways, which then signal prosurvival events, as has been shown by knockdown of hKSR2 expression (31). The prosurvival events include upregulation of the antiapoptotic Mcl-1 (30) and altered Bcl-2/Bad ratios (31). Here, we document a role for miR-32 in the prosurvival events, but its relationship to the other signaling members of apoptosis/survival network remains to be elucidated.

The exquisite cell context specificity of miRNA targets is well known, and any given miRNA may be targeted by different miRNAs in different cell types or even cell subtypes. (e.g., refs. 38, 39). It is therefore important to note that in both HL60 and U937 cells, which belong to different FAB subtypes, miR-32 targets Bim (Fig. 4). Also, recent studies in the Croce laboratory have shown that in 2 subtypes of cultured human prostate cancer cells, miR-32 inhibits the expression of Bim by a 3′-UTR–mediated mechanism (25). Taken together with results reported here, this suggests that the miR-32–Bim relationship is of wide significance in human cells. This contrasts with the targeting of p27Kip1 by miR-181 previously reported in AML cells (23, 40), whereas in a variety of solid tumors p27Kip1 is targeted by miR-221/222 (41–43). These examples show that miRNA targets need to be established 1 cell type at a time.

Currently, there are only a few published reports of the ability of 1,25D to regulate key cellular functions through modulation of miRNA expression. Previously, we showed that an exposure of human AML HL60 and U937 cells to 1,25D results in downregulation of the miR-181 family, with the most prominent effect on miR-181a (23). This was associated with the upregulation of p27Kip1 expression and contributed to 1,25D-induced cell-cycle arrest. More recently, miR-24 was reported to be upregulated by 1,25D and related to diminished cell proliferation (44), and a role for miRNA in cell-cycle control by 1,25D was described in cultured nonmalignant RWPE-1 human prostate epithelial cells (45). In RWPE-1 cells 1,25D upregulates the DNA helicase MCM7 gene, in which the miR-106 is embedded in intron 13 (46, 47), which then targets p21/CDKN1A and contributes to cell-cycle arrest. Thus, this article provides a new aspect of the accumulating evidence that miRNAs participate in the critical aspects of vitamin D...
regulation of the essential cellular processes, such as those controlling the cell cycle, cell survival, and cell differentiation.

Selective upregulation of miR-32 in at least some human cancers, including prostate carcinoma and multiple myeloma, (25, 48) seems to play a role in malignant transformation by providing survival advantage to cells with high expression of miR-32 and reduced levels of Bim. A similar mechanism described here seems to aid the phagocytic function of monocytes, which produces intracellular stress as a consequence of generation of ROS needed to dispose of phagocytized material. But because increased survival capacity is a disadvantage when malignant cells are to be eradicated by cytotoxic agents, consideration should be given to the design of differentiation therapy regimens in which compounds or conditions which reduce the expression of miR-32, or increase

the levels of Bim (49, 50), are administered along with the therapeutic agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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