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MicroRNAs181 regulate the expression of p27^{Kip1} in human myeloid leukemia cells induced to differentiate by 1,25-dihydroxyvitamin D_3

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Abstract

Human myeloid leukemia cells exposed to 1,25-dihydroxyvitamin D_3 (1,25D), a major cancer chemopreventive agent, acquire features of normal monocytes and arrest in the G_1 phase of the cell cycle, due to the upregulation of p27^{Kip1} and p21^{Cip1}, but the mechanism is not clear. Here evidence is provided that an exposure of HL60 and U937 cells to low (1–10 nM) concentrations of 1,25D decreases the expression of miR181a and miR181b in a concentration and time-dependent manner. Since the predicted miR181 targets include the 3'-UTR of p27^{Kip1}, we expressed pre-miR181a in these cells, and found that the elevation of cellular miR181a levels abrogates the 1,25D-induced increase in p27^{Kip1} at both mRNA and protein levels. In contrast, transfection of pre-miR181a resulted in a slight elevation of p21^{Cip1} expression. Importantly, transfection of pre-miR181a blunted the effect of 1,25D on the expression of monocytic differentiation markers, and reduced the G_1 block in 1,25D-treated cells, while transfection of anti-miR181a increased 1,25D-induced differentiation. Together, these data show that miR181a participates in 1,25D-induced differentiation of HL60 and U937 cells, and suggest that a high constitutive expression of members of miR181 family may contribute to the malignant phenotype in the myeloid lineage.

Keywords

MicroRNA 181; vitamin D; p27^{Kip1}; p21^{Cip1}; myeloid leukemia; differentiation

Introduction

One of the unsolved puzzles in hematopoiesis is how cell differentiation is coupled to the retardation, and then cessation, of proliferative activity. The practical importance of this coupling is that impaired differentiation leads to uncontrolled cellular proliferation and its attendant genetic instability results in malignancy. Thus, studies of the reversal of the malignant phenotype are likely to provide clues to the mechanistic basis for the differentiation-growth arrest connection.

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Among cellular systems used for such studies, the promyeloblastic leukemia HL60 and pro-monocytic leukemia U937 cells have been widely utilized, and the physiological derivative of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25D) can induce (HL60 cells) or enhance (U937 cells) the monocytic phenotype and arrest their proliferation, predominantly in the G_1 phase of the cell cycle.[1–4] Important mechanistic insights were obtained when it was reported that the expression of $p27^{Kip1}$ and $p21^{Cip1}$, the small protein inhibitors of cyclin-dependent kinases (Cdks), is upregulated by 1,25D in both HL60 and U937 cells,[5–7] and that in the case of $p27^{Kip1}$ this is largely the result of the stabilization of this protein due to reduced expression of subunits of S-phase kinase protein-2 (Skp2).[8] Further studies demonstrated that $p27^{Kip1}$ degradation is also controlled by the cyclin-dependent subunit 1 (Cks1),[9] and by Spy1.10 In addition, $p27^{Kip1}$ can be inactivated by cytoplasmic sequestration,[11] or downregulated by miR221/222 in a number of solid tumors.[12–14]

The evidence presented here demonstrates that in cultured human myeloid cells the expression of $p27^{Kip1}$ can also be regulated by at least two members of miR181 family, and that 1,25D-induced repression of these microRNAs contributes to the accumulation of $p27^{Kip1}$ and the arrest of the cell cycle progression in G_1 phase.

Results

Exposure of HL60 and U937 cells to 1,25D downregulates the expression of miR181a and miR181b in a dose and time-dependent manner

MicroRNAs repress protein expression at the posttranscriptional level, and are coming into prominence as regulators of most biological functions, including differentiation and hematopoiesis. For instance, retinoic acid downregulates the expression of miR181b in APL cells. Using a miRNA microarray platform[21] we found that the principal miRNAs downregulated by 1,25D in HL60 cells were several members of the miR181 family, with the most markedly downregulated one being miR181a (data not shown).

The dose-dependent decreases in miR181a and miR181b were confirmed by qRT-PCR (Fig. 1A), and the downregulation of these miRs was also time-dependent (Fig. 1B and C), as was 1,25D-induced downregulation of miR181c (not shown). The 1,25D-induced decreases in miRs181 were less marked in U937 than in HL60 cells, possibly due to the later stage of the arrest of hematopoietic maturation of U937 cells, with cellular programs for monocytic phenotype already partially in place.

The 3'-UTR of $p27^{Kip1}$ has a potential miR-181 binding site

Public web-based prediction sites were used to predict target sites in the 3' untranslated regions (3'-UTR) of human gene transcripts for possible binding of miRNAs, e.g., microRNA. org, 16 and TargetScan.17 In addition, three target prediction online softwares (MIRANDA, TARGETSCAN and PICTAR-VERT) are available for miR181a at Sanger miRBase. As indicated in Figure 2, there is a potential miR181 binding site in the 3'-UTR of $p27^{Kip1}$ gene,[16,17] which shares homology with the mouse $p27^{Kip1}$ gene. The alignment scores with the human gene are: 181a= 158; 181b= 150; 181c = 148; and 181d = 145.[16,17] No potential binding sites for miRs181 in the p21Cip1 gene were reported.[16,17]

Transfection of pre-miR181a abrogates 1,25D-induced upregulation of $p27^{Kip1}$, but not $p21^{Cip1}$ gene

Since miR181a has the best predicted alignment with the $p27^{Kip1}$ target site, the subsequent experiments focused on this miR. When exogenous pre-miRs are expressed in leukemia cells they are processed to functional miRs.[22] We first confirmed the efficiency of the transfection procedure by transfecting a test miRNA, pre-miR1, and found that compared to the non-

targeting miR, it reduced the levels of protein tyrosine kinase 9 (PTK9) mRNA, the target of miR1, to approximately 30% of the control level (data not shown). Direct measurement of miR181a levels in HL60 and U937 cells transfected with pre-miR181a and treated with 1,25D showed that miR181a levels approximately doubled (Fig. 3).

The results of pre-miR181a transfections in HL60 and U937 cells on the $p27^{Kip1}$ target are shown in Figure 4A and B. In HL60 cells there was a marked reduction in $p27^{Kip1}$ mRNA and protein levels compared to non-target miR transfection, and less marked decreases in U937 cells were also observed. However, there appeared to be no significant decrease in either $p21^{Cip1}$ mRNA (Fig. 4A) or protein levels (Fig. 4B) following pre-miR181a transfection. These results show that in 1,25D-treated myeloid leukemia cells $p27^{Kip1}$ is a specific target of miR181a, in contrast to the situation in solid tumors such as glioblastoma, hepatocellular carcinoma, and thyroid carcinoma, in which miRs221/222 have this role.[12–14] However, it is also possible that in other circumstances miRs181 and 221/222 can act in concert.[23]

Transfection of pre-miR181a inhibits 1,25D-induced expression of monocytic differentiation markers and G_1 arrest

The modulation by miR181a of the 1,25D effect on leukemia cell differentiation marker CD14 appears to parallel the downregulation of p27 mRNA levels in both HL60 and U937 cells (compare Fig. 5A with Fig. 4A). The reduction in the surface marker CD11b was less marked, and no difference was noted in the basal levels of either marker, consistent with little change in miR181a RNA levels in cells not treated with 1,25D. This suggests that the principal action of the transfected miR181a occurs in the context of 1,25D-induced cellular alterations, and that threshold levels of miR181a are necessary for the biological effects.

The 1,25D-induced G_1 cell cycle arrest in HL60 cells was also markedly reduced by pre-miR181a transfection (Fig. 5B), but in an experiment performed in parallel 1,25D did not induce a significant G_1 block in U937 cells. However, the small G_1 block apparent in U937 cells in Figure 5B, was consistently seen in individual experiments, and was reduced when pre-miR181a was transfected (not shown).

MiR181a inhibitor enhances differentiation induced by low concentration of 1,25D

To further confirm that miR181a is a negative regulator of monocytic differentiation, we transfected miR181a inhibitor, as well as non-targeting microRNA, and treated HL60 cells with 1 nM 1,25D for 48 h. Determination of surface markers of differentiation showed that in cells transfected with miR181a antagonist oligo CD14 expression increased by 23.9% compared to control transfection , CD11b increased by 25.0%, and the measurements of cell cycle parameters showed a 22.2% increase in the G_1/S phase ratio, indicating that inhibition of miR181a expression accelerates monocytic differentiation.

Discussion

Vitamin D is a major cancer chemopreventive agent, but the mechanisms of its actions are not well understood. The studies presented here demonstrate that miRs181 contribute to the control of G_1 to S phase transition in myeloid leukemia cells differentiating when treated with 1,25D. This is a new role for miRs181, previously linked to the B-lineage cells in mouse bone marrow in which it is preferentially expressed.[24] In murine cells miR181a was also found to have increased expression in mature T cells, where it acts as a “rheostat” which governs T cell sensitivity to antigens.[25]

The current report provides the first evidence for an involvement of miR181 in the modulation of the expression of the cell cycle regulator $p27^{Kip1}$. It is clear that miRs181 contribute to the

control of G_1 to S phase transition in myeloid leukemia cells differentiating when treated with 1,25D. It appears that in proliferating HL60 cells the constitutively high expression of miRs181 results in low levels of $p27^{Kip1}$ mRNA and protein, which are insufficient to inhibit Cdk4/6 activity, and thus the cells continue to traverse the cell cycle. When 1,25D is added, miRs181 levels are reduced, resulting in increases at first in $p27^{Kip1}$ mRNA, then $p27^{Kip1}$ protein levels, contributing to the G_1 block. It is not surprising that when the levels of miR181a are artificially increased by the transfection of exogenous pre-miR181a there is only a modest, although significant ($p < 0.05$), reversal of the cell cycle block, since $p21^{Cip1}$ is also involved in the G_1 block, and $p21^{Cip1}$ levels are increased by 1,25D.[5,7] Further, protein levels of $p27^{Kip1}$ are also regulated by its phosphorylation on S10,[26] and T198,[27] which stabilize $p27^{Kip1}$, and on T187,[28] which is followed by Skp2-dependent ubiquitination,[8,29] with involvement of Cks1,[9] and Spy1.[10] Clearly, the importance to the cell survival of a smooth G_1 to S phase transition requires a concerted action of several mechanisms, and an interference with only one such mechanism may have only a moderate effect. Thus, it would not be realistic to expect a complete control of p27 levels by miRs181 alone.

It is also interesting that retinoic acid, as mentioned above, has been reported to downregulate the expression of miR181b in Acute Promyelocytic Leukemia cells, though the effects on the other miRs, 181a, 181c and 181d, and the link between the downregulation of miR181b and cell cycle control were not addressed in that study.[21] Nonetheless, the Garzon et al. report[21] and the present study combine to demonstrate that the downregulation of miRs181 is important for the induced differentiation of myeloid leukemia cells. These studies also illustrate the exquisite cell context-specificity of microRNA expression and their functional targets,[30] and support the possibility that enhanced miR181 expression contributes to the initiation of the neoplastic changes that lead to myeloid leukemias.

Materials and Methods

Chemicals and antibodies

1,25D was a kind gift from Dr. Milan Uskokovic (Bioxell, Nutley NJ). Complete protease inhibitor cocktail was purchased from Hoffmann-La Roche (Nutley, NJ). For Western blotting studies, antibodies p21, p27 and Crk-L, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit and anti-goat antibodies linked to HRP were purchased from Cell Signaling Technologies (Danvers, MA).

Cell culture

HL60-G cells, derived from a patient with promyeloblastic leukemia,[15] were cultured in suspension in RPMI-1640 medium supplemented with 10% bovine calf serum (Hyclone, Logan, UT) in a humidified atmosphere containing 5% CO_2 at 37°C. U937 cells obtained from ATCC (Manassas, VA) were cultured under the same conditions as HL60 cells, though the concentration of 1,25D routinely used was 10 nM, as U937 cells are less sensitive to 1,25D than HL60 cells, which unless indicated otherwise, were treated with 1 nM 1,25D. Cells were passaged 2–3 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 suspended in fresh medium and the experiment was repeated at least three times.

MicroRNA target predictions

Public web-based prediction sites were used to predict target sites in the 3' untranslated regions (3'UTR) of human gene transcripts for possible binding of miRNAs, e.g., microRNA.org resource (http://www.microrna.org/microrna),[16] and TargetScan (http://www.targetscan.org).[17] In addition, and as example, three target prediction online softwares (MIRANDA,

TARGETSCAN and PICTAR-VERT) are available for miR181a at Sanger miRBase of http://microrna.sanger.ac.uk/cgi-bin/sequences/mirna_entry.pl?acc=MI0000289.

Transfection of miRNA precursors and inhibitors

This was carried out using Endo-Porter delivery reagent from Gene Tools Inc., (Philomath, OR). Pre-miR181a and non-targeting pre-miR miRNA precursors (Ambion, Austin, TX) were transfected at a final concentration of 10 nM for 24 hr before exposure to other compounds. Pre-miR-1 miRNA precursor was designed for use as a positive control in the optimization of transfection conditions. This positive control can effectively downregulate the expression of PTK-9 at the mRNA level.[18] Anti-miR181a or anti-miR Negative Control #1 (Ambion) were transfected at a final concentration of 10 nM for 24 hr before exposure to other compounds.

Markers of monocytic differentiation

Aliquots of 1×10^6 cells were harvested, washed twice with phosphate buffered saline (PBS), suspended and incubated for 45 min at room temperature with 0.5 μl MY4-RD-1 and 0.5 μl MO1-FITC antibodies to analyze the expression of surface cell markers CD14 and CD11b, respectively. The cells were then washed three 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 analysis

The DNA content of HL60 cells was determined as follows: one million cells were harvested and washed twice with phosphate buffered saline (PBS), then fixed with 75% ethanol at −20° C for 24 hr. Cells were then collected and resuspended in 1 ml of PBS with RNase (at 10 μg/ml, Sigma) and propidium iodide (PI at 10 μg/ml, Sigma) for 30 min. at 37°C. PI stained cells were analyzed using EPICS flow cytometer. The resultant histogram of DNA content was gated and analyzed using the multicycle program to determine the proportions of cell cycle.

RNA extraction, miRNA expression profiling and qRT-PCR confirmation

Total RNA containing microRNA was extracted by using Trizol (Invitrogen, CA) according to manufacturer's protocol. miRNA expression profiling was performed on OSUCCC version 4.0 arrays (ArrayExpress Accession # A-MEXP-1246) and the methodology of miRNA profiling was as described.[19] In brief, 2.5–5 μg of total RNA was reverse transcribed into first strand cDNA using biotin end-labeled random octamer oligo primers. The biotin-labeled cDNA targets hybridize to probes on array and the hybridization signal was further amplified and detected using Streptavidin Alexa 647 (Invitrogen). The assayed arrays were scanned by Axon 4000B (Molecular Device, CA) using GenePix 6.0 software. The miRNAs expressed differentially on the array were further validated and confirmed using qRT-PCR as described here. cDNA was synthe using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Mature miRs were quantitated using two-step TaqMan real-time PCR with TaqMan microRNA kit for each microRNA studied here. MiR181 expression levels shown here were normalized using U6 rRNA (Applied Biosystems). In preliminary experiments normalization using 18S RNA also showed similar results.

Quantitative Real Time PCR for $p21^{Cip1}$ and $p27^{Kip1}$ was carried out using a lightcycler with FastStart DNA SYBR Green PCR kit (Roche Diagnostics, Indianapolis, IN) as described before.[20] 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 p21 were: upstream 5'-TTA GCA GCG GAA CAA GGA GTC AGA-3', downstream 5'-ACA CTA AGC ACT TCA GTG CCT CCA-3'; primers for p27 were upstream 5'-AGC AAT GCG CAG GAA TAA GGA AGC-3', downstream 5'-TAC GTT TGA CGT CTT CTG AGG CCA-3'. For RNA Pol

II the primers were: upstream 5'-GCA CCA CGT CCA ATG ACA T-3', downstream 5'-GTG CGG CTG CTT CCA TAA-3'. The quality of PCR product was monitored using post-PCR melting curve analysis.

Western blotting

Western blotting was performed using whole cell extracts as described before.[20] Briefly, membranes were incubated overnight with different primary antibodies, and then blotted with a horseradish peroxidase-linked secondary antibody for 1 hr. The protein bands were visualized using a chemiluminescence assay system (Pierce Biotechnology, Rockford, IL), each membrane was stripped and reprobed for internal control Crk-L. The optical density of each band was quantitated using ImageQuant 5.0 (Molecular Dynamics, Sunnyvale, CA).

Statistical methods

Each experiment was performed at least three times and the results were expressed as percentages (mean ± SE) of the vehicle controls. Significance of the differences between mean values was assessed by a two-tailed Student's t-test. All computations were performed with an IBM-compatible personal computer using Microsoft EXCEL.

Abbreviations

miR	microRNA
1,25D	1,25-dihydroxyvitamin D_3
UTR	untranslated region

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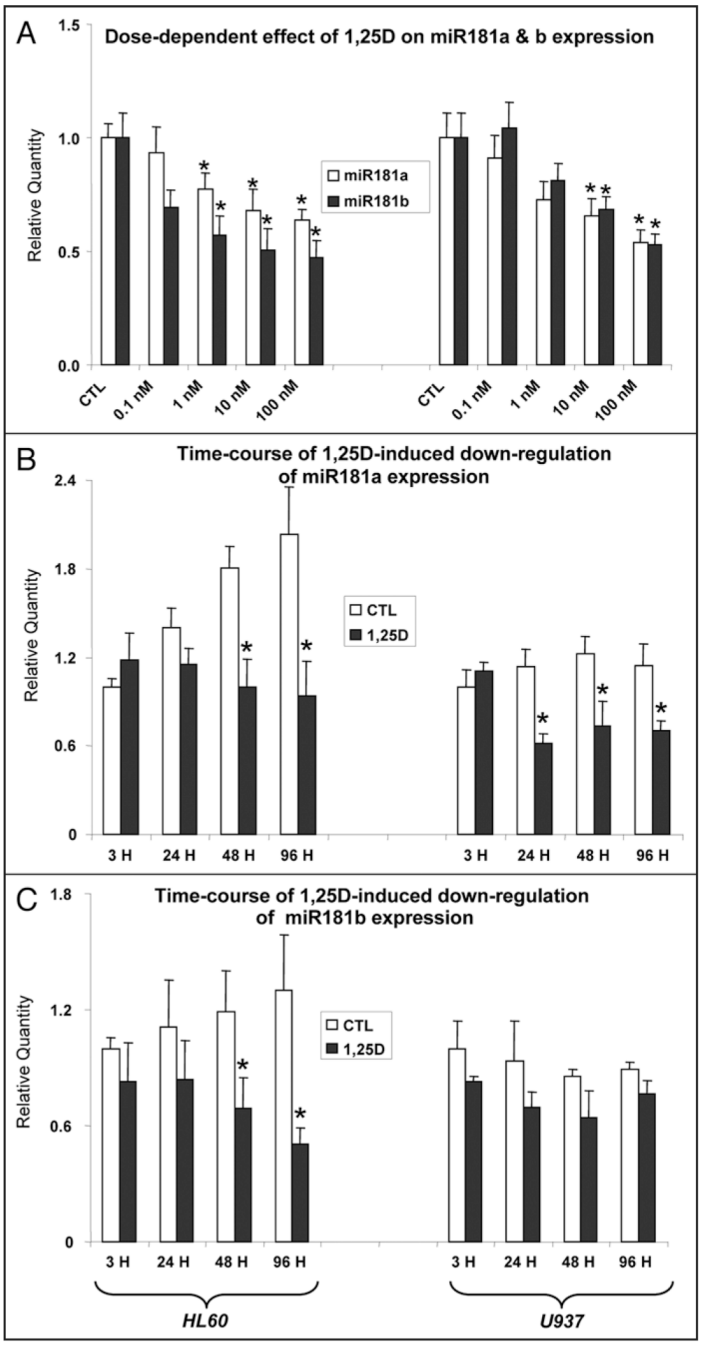

Figure 1.

Dose and time-dependent downregulation of miR181a and miR181b expression determined by qRT-PCR. (A) Mir181 levels after an exposure for 48 hr (H) to the indicated concentrations of 1,25D of HL60 (left), or U937 cells (right). (B) Time-dependent decrease in miR181a following an exposure to 1 nM (HL60) or 10 nM (U937) cells, which are less sensitive to 1,25D. (C) As (B), but levels of miR181b are shown here. Asterisks show the statistically significant differences from the corresponding vehicle-treated control (CTL) ($p < 0.05$; mean values +/− SE, n = 3).

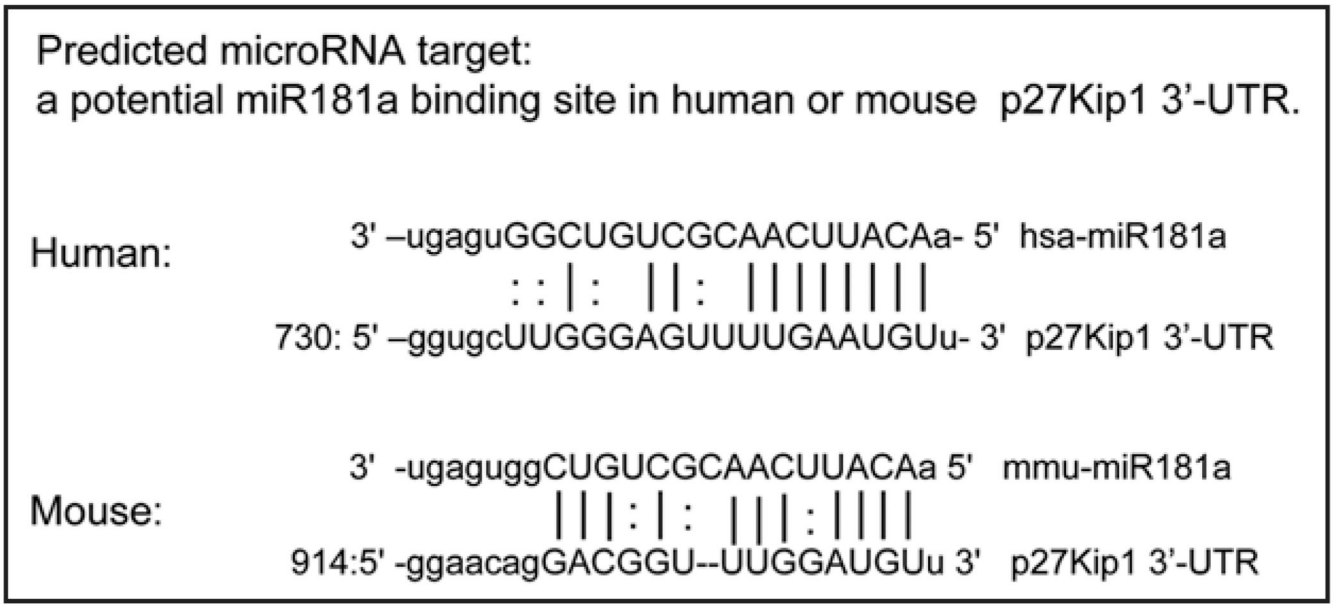

Figure 2.
The predicted miR181a binding site in $p27^{Kip1}$ 3'-UTR. Schematic representation of potential miR181a binding sites within the 3'-UTR of human and mouse $p27^{Kip1}$ genes.

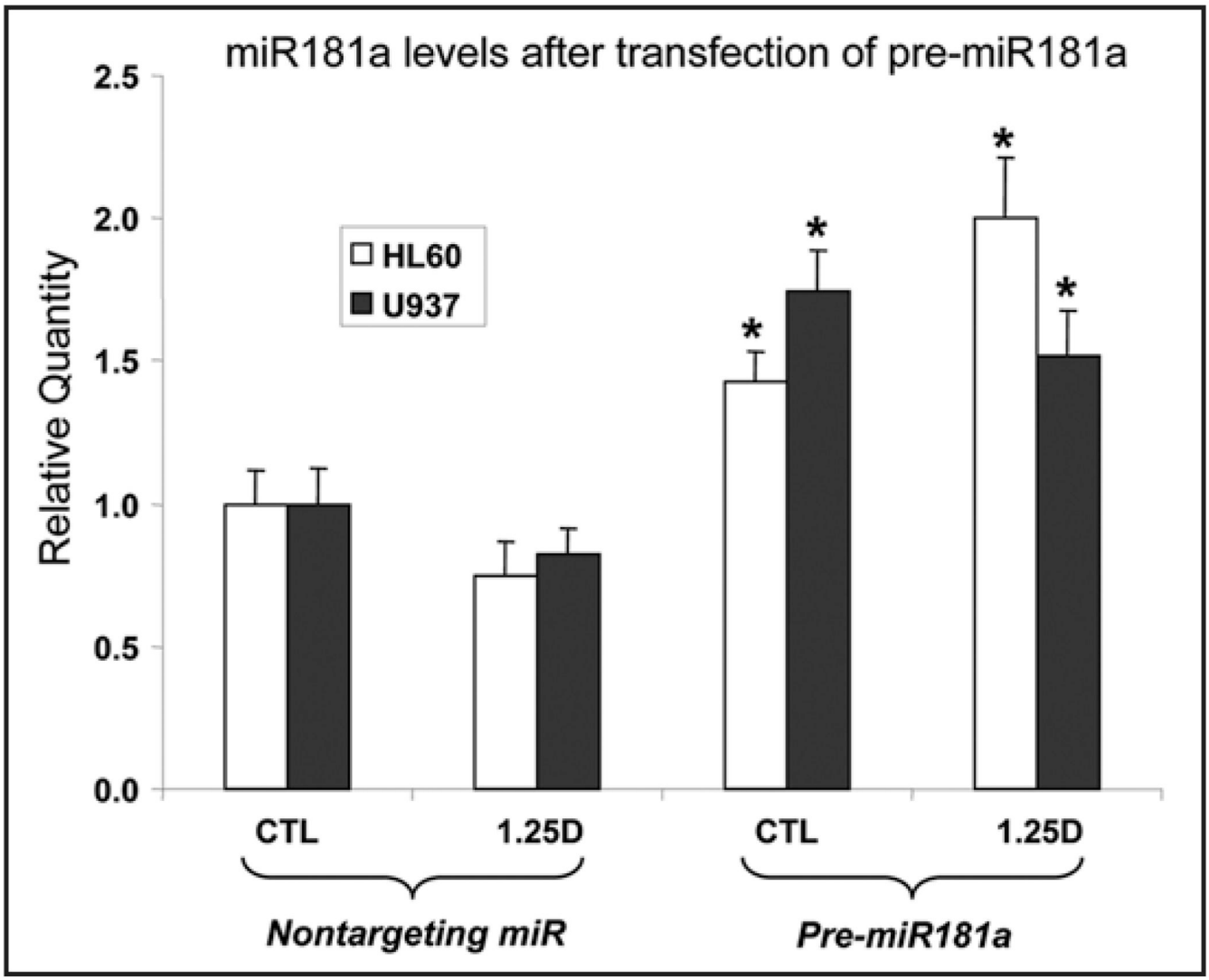

Figure 3.
Cellular levels of miR 181a following transfection of HL60 cells with pre-miR181a or the nontargeting (NT) control miR, and 24 hr later addition of ethanol vehicle or 1 nM 1,25D for additional 48 hr. Asterisks show the statistically significant differences from the corresponding NT miRNA-treated control ($p < 0.05$; mean values +/− SE, n = 3).

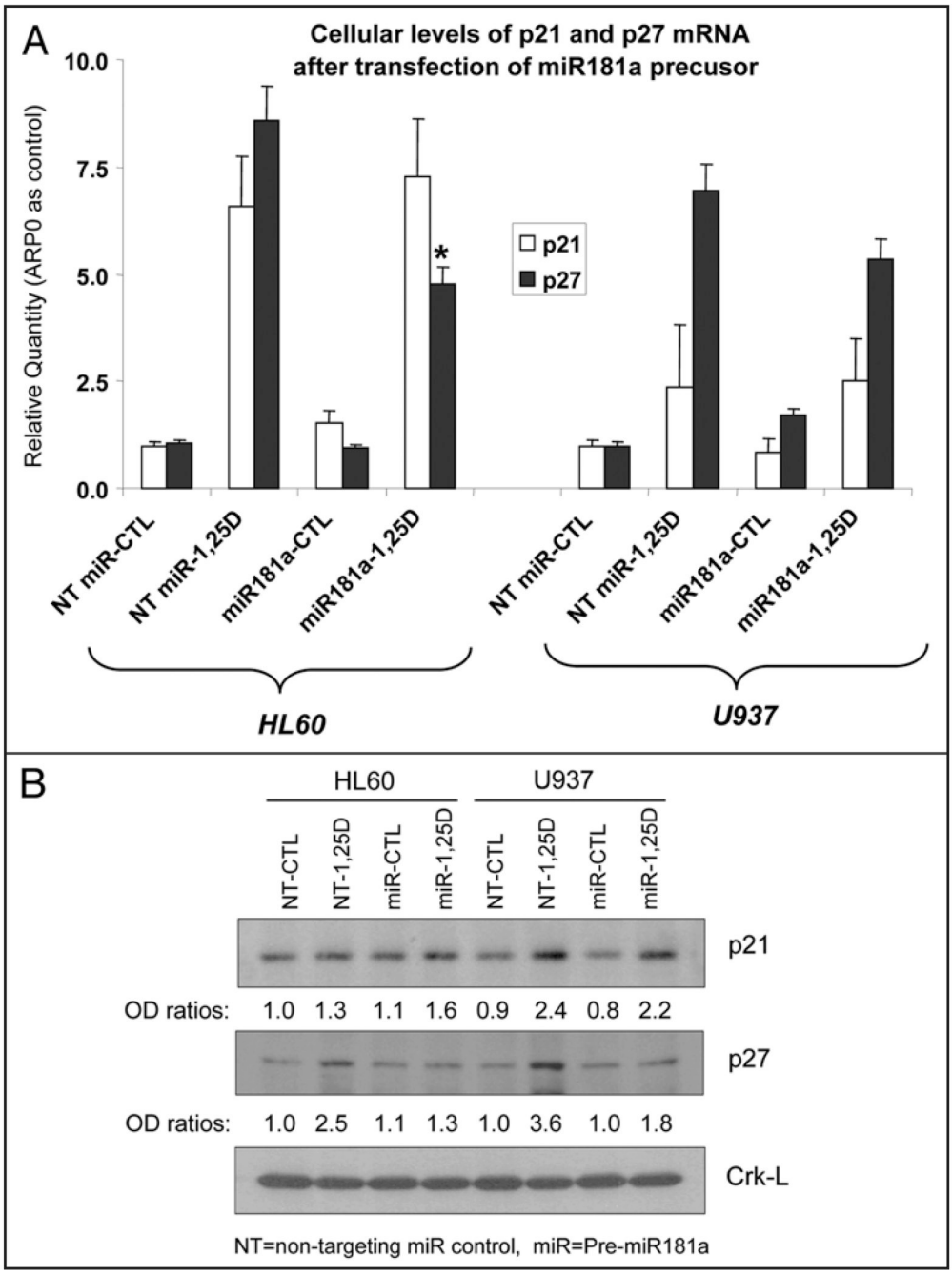

Figure 4.

Transfection of pre-miR181a abrogates 1,25D-induced upregulation of $p27^{Kip1}$. (A) Levels of $p27^{Kip1}$ and $p21^{Cip1}$ mRNA determined by qRT-PCR. The asterisk shows the statistically significant difference from the corresponding non-targeting miRNA-treated control ($p < 0.05$; mean values +/− SE, n = 3). (B) Levels of $p27^{Kip1}$ and $p21^{Cip1}$ protein determined by Western blotting. The concentration of 1,25D was 10 nM, treatment for 48 hr. NT = non-targeting miR control; miR=pre miR 181a. Protein levels of Crk-L were loading controls, and were used to normalize the optical density ("OD ratios") of each band shown below each panel. The blots shown are representative of three experiments.

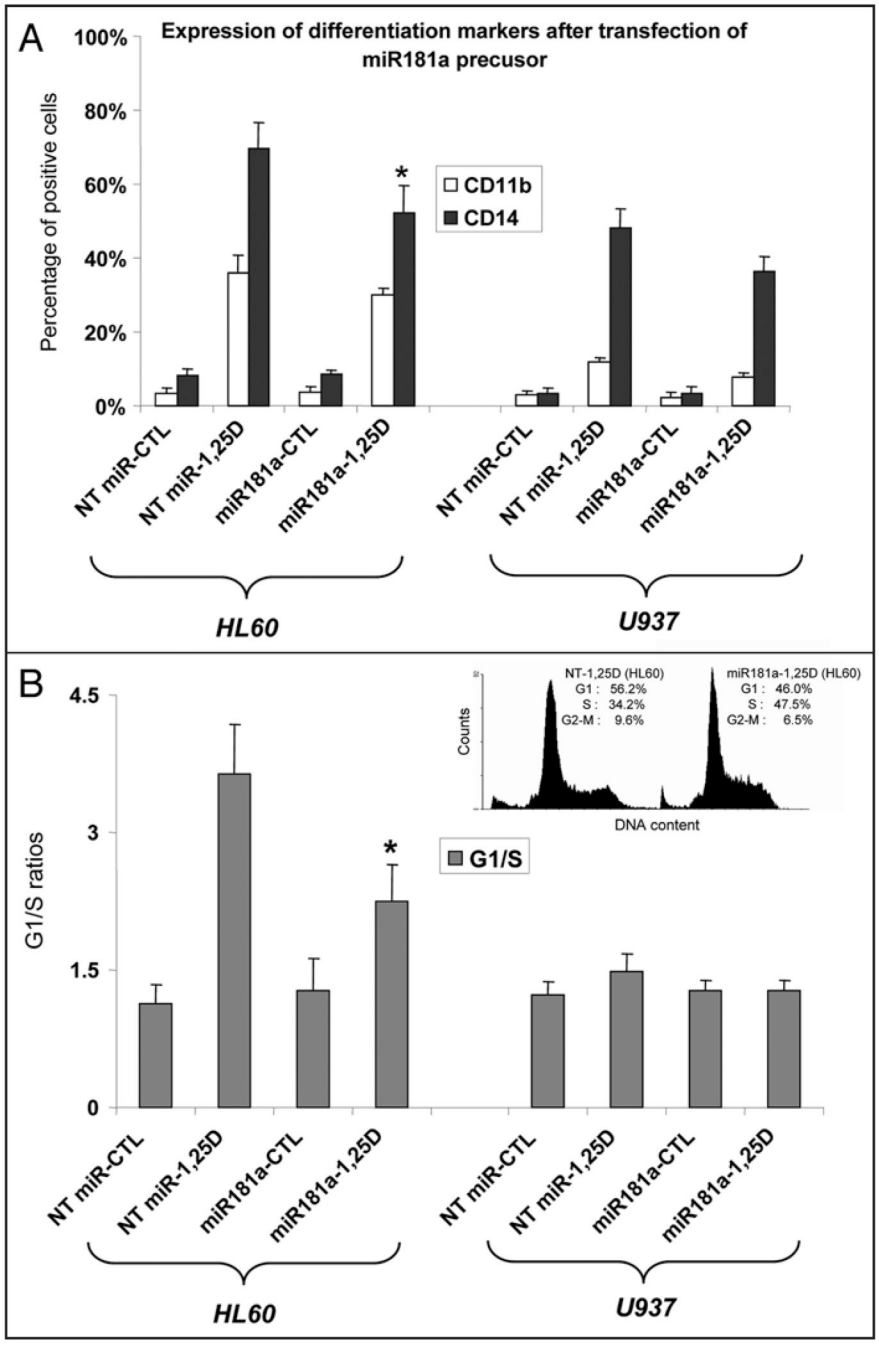

Figure 5.

Inhibition of 1,25D-induced differentiation and cell cycle block by miR181a. (A) Effect of miR181a on the expression of differentiation markers CD11b and CD14. 10 nM 1,25D was used for 48 hr. (B) Effect on the G_1 to S phase ratios of the proportion of cells in each compartment, determined by flow cytometry. The insets in panel B show examples of the abrogation of 1,25D-induced G_1 to S phase block by transfection of pre-miR181a in HL60 cells. (In control cells G_1 was 46.2%, S phase was 38.0%; cells exposed only to miR181a: G_1 was 42.5%, S phase was 48.8%. For comparison, the values shown in the inset for 1,25D-treated cells were G_1: 56.2%, S phase: 34.2%, while miR181a transfection and treatment with 1,25D, the values were G_1: 46.0%, S phase: 47.5%). Asterisks show the statistically significant

differences from the corresponding non-targeting (NT) miRNA-treated control ($p < 0.05$; mean values +/− SE, n = 3).