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## MicroRNA-32 upregulation by 1,25-dihydroxyvitamin D<sub>3</sub> in human myeloid leukemia cells leads to Bim targeting and inhibition of AraC-induced apoptosis

Elzbieta Gocek<sup>1,2</sup>, Xuening Wang<sup>1</sup>, Xiuping Liu<sup>3</sup>, Chang-Gong Liu<sup>3</sup>, and George P. Studzinski<sup>1,\*</sup>

<sup>1</sup>Department of Pathology and Laboratory Medicine, UMD-New Jersey Medical School, Newark, New Jersey, 17101-1709, USA <sup>2</sup>Faculty of Biotechnology, University of Wroclaw, Tamka 2, 50-137 Wroclaw, Poland <sup>3</sup>Department of Experimental Therapeutics – Unit 36, Center for Targeted Therapy, The University of Texas-MD Anderson Cancer Center, Texas, 77030, USA

### Abstract

1,25-dihydroxyvitamin D<sub>3</sub> (1,25D) used to treat human acute myeloid leukemia (AML) cells induces features of normal monocytes, but the mechanisms underlying this response is not fully understood. We hypothesized that one or more microRNAs (miRNAs) 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, where it targets the 3'-UTR of the mRNA encoding the pro-apoptotic 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 pre-exposed 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.

### Keywords

MicroRNA-32; Vitamin D Differentiation; Bim; Acute Myeloid Leukemia; AraC; Apoptosis

\*Corresponding author: George P. Studzinski, MD, Ph.D. Department of Pathology and Laboratory Medicine, UMD-New Jersey Medical School, 185 South Orange Ave, Newark, New Jersey, 07101-1709, USA Tel: (973) 972-5869, Fax: (973) 972-7293, studzins@umdnj.edu.

## Introduction

Acute myeloid leukemia (AML) is a hematological 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 supra-physiological concentrations of the active form of vitamin D, 1,25-dihydroxyvitamin D<sub>3</sub> (1,25D) (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 (TFs), e.g. 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, while 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 pro-differentiation activity ATRA also promotes cell death, and is particularly effective when combined with arsenic trioxide (As<sub>2</sub>O<sub>3</sub>), 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 if 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 pro-apoptotic protein Bim (14, 15). Therefore, we investigated if the increased levels of miR-32 in 1,25D-treated AML cells can be validated by qRT-PCR, and if so, if miR-32 regulates the expression of Bim, and whether this is 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 ATCC (Manassas, VA) were cultured in suspension in RPMI-1640 medium supplemented with 10% bovine calf serum (Hyclone, Logan, UT) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. For U937 cells concentration of 1,25D routinely used was 10 nM, as these cells are less sensitive to 1,25D than HL60 cells, which 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 resuspended in fresh medium and each experiment was repeated at least three times.

### **Isolation of mononuclear cells from peripheral blood and selection of monocytes**

Peripheral blood samples were obtained from five healthy volunteers according to institutional IRB protocol. Mononuclear cells were isolated by using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO), as previously described (17). Monocytes were positively selected with CD14 MicroBeads (Miltenyi Biotec Inc. Auburn, CA) as directed by manufacturer's protocol. Homogeneity of CD14 positive cells was determined by using EPICS XL flow cytometer (Beckman Coulter, Miami, FL).

### **Chemicals and antibodies**

1,25D was a kind gift from Dr. Milan Uskokovic (Bioxell, Nutley NJ). Antisense oligonucleotides against Drosha (Cat#L-016996-00) and Dicer (Cat#L-003483-00) were obtained from Thermo Scientific (Chicago, IL), hsa-miR-32 anti-miR miRNA inhibitor (ID: AM12716) and hsa-miR-32 pre-miR miRNA precursor (ID: PM12716) were obtained from Applied Biosystems (Foster City, CA). Complete protease inhibitor cocktail was purchased from Hoffmann-La Roche (Nutley, NJ). Antibodies were procured from Cell Signaling Technologies, Denver, MA (Bim, Bax, secondary anti-rabbit, and anti-mouse linked to HRP), and from Santa Cruz Biotechnology, Santa Cruz, CA (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 microcosm 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, 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) performed 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. (Philomath, OR). 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, Austin, TX) and appropriate non-targeting control oligonucleotides were transfected at a final concentration of 20 nM for 48 h before exposure to other compounds.

### RNA isolation and quantitative RT-PCR

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

Quantitative RT-PCR for Bim was carried out using a FastStart DNA SYBR Green PCR kit (Roche Diagnostics, Indianapolis, IN) as described before (23). Fold changes of mRNA levels in target gene relative to the RNA polymerase II (RP II) control were calculated by relative quantification analysis. Primers used for Bim were: upstream 5'-AGTTCTGAGTGTGACCGAGAAGGT-3', downstream 5'-TCCTGCTTGTGGCTCTGTCTGT A-3'; for RP II, upstream 5'-GCACCACGTCCAATGACAT-3', downstream 5'-GTGCGG CTGCTTCCATAA-3'. The quality of PCR products were monitored using post-PCR melting curve analysis.

### Markers of monocytic differentiation

Aliquots of  $1 \times 10^6$  cells were harvested, washed twice with phosphate buffered saline (PBS) and incubated for 45 min at room temperature with 0.5  $\mu$ l MY4-RD-1 and 0.5  $\mu$ l MO1-FITC antibodies (Beckman Coulter Inc, Brea CA) to analyze the expression of monocytic differentiation surface 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 distribution

The DNA content of the cells was determined as follows:  $1 \times 10^6$  cells were harvested and washed twice with PBS, then fixed with 75% ethanol at  $-20^\circ\text{C}$  for 24 h. Cells were then collected and resuspended in 1 ml of PBS with 10  $\mu$ g/ml RNase (Sigma) and 10  $\mu$ g/ml propidium iodide (Sigma) for 30 min at  $37^\circ\text{C}$ . 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 non-viable cells.

### Annexin V and propidium iodide staining

AML cells were induced to apoptosis by 100  $\mu$ M AraC (Sigma) for 24 h. Samples were collected, washed once with PBS and resuspended in the 10 mM HEPES/NaOH binding buffer, containing 0.14 M NaCl and 2.5 mM  $\text{CaCl}_2$ , pH 7.5. Apoptotic cells were stained using an Annexin V-FITC Kit (Sigma). Cells were incubated with 50  $\mu$ g/ml Annexin V and 20  $\mu$ g/ml propidium iodide in the dark, at room temperature for 10 min, and immediately analyzed by flow cytometry (EPICS XL). Cells Annexin V-positive/PI-negative were considered as early apoptotic, cells doubly positive, as late apoptotic.

## Western blotting

Western blotting was performed 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 h. 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 or calregulin). 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. The results were expressed relative to vehicle controls, or as percentages of the cell population (mean  $\pm$  SE). 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.

## Results

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

The initial experiments were performed using a miRNA microarray platform (24) to identify the expression of miRNAs after exposure to 1,25D. Ingenuity Pathway analysis indicated that only miR-32 was differentially expressed in both HL60 and U937 1,25D-treated cells. As shown in Fig 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 Fig 1B, demonstrated 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. While Taq-Man 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 pro-apoptotic protein Bim is a potential target of miR-32 in AML cells

In silico studies reported in at least six public searches (Fig 2A) revealed that 3'UTR of BCL2L11 gene, which encodes the pro-apoptotic 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). While 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 if 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 Fig 3A, transfection of silencing constructs of Dicer and Drosha effectively abrogated 1,25D-induced up-regulation of miR-32 expression, though the ambient levels of miR-32 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), while 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 down-regulation of Bim can be reversed by anti-miR-32. In contrast, the levels of Bax, another pro-apoptotic protein, are essentially unaltered by the manipulation of miR-32 cellular levels (Fig 4, C and D).

### Precursor miR-32 promotes 1,25D-induced AML cell differentiation while 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), while 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 Fig 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 pre-incubating AML cells for

48 h with anti-miR-32, then treating the cells with 100  $\mu$ M AraC for 24 h, 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 three sets of determinations showed that anti-miR-32 increases cell death induced by AraC (Table 1A, groups 3 and 4, 7 and 8) and abrogates the previously reported anti-apoptotic effect of 1,25D (31). The protective effect of 1,25D is seen here for HL60 cells (Table 1A, 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 1B). In the experiments where 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 1B). This is perhaps seen because other components of cell survival network play a greater role than Bim when pre-miR-32 reduces Bim levels, while increased Bim levels in cells treated with anti-miR-32 have a more dominant effect on cell survival. Taken together, the two 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 miR32 levels, such as anti-miR32.

## 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.

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 and AKT pathways (33–36), though the specific details regarding the sequence of molecular events are few. One possibility is that the hKSR2 gene, which is directly up-regulated by 1,25D (37), provides an upstream platform for activation of MAPK pathways, which then signal pro-survival events, as has been shown by knock-down of hKSR2 expression (31). The pro-survival events include up-regulation of the anti-apoptotic Mcl-1 (30), and altered Bcl-2/Bad ratios (31). Here, we document a role for miR-32 in the pro-survival events, but its relationship to the other signalling members of apoptosis/survival network remains to be elucidated.

The exquisite cell context specificity of miRNA targets is well known, and any given mRNA may be targeted by different miRNAs in different cell types or even cell subtypes. (e.g. (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 two 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 one 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 down-regulation of the miR-181 family, with the most prominent effect on miR-181a (23). This was associated with the up-regulation of p27Kip1 expression, and contributed to 1,25D-induced cell cycle arrest. More recently, miR-24 was reported to be up-regulated 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 non-malignant RWPE-1 human prostate epithelial cells (45). In RWPE-1 cells 1,25D up-regulates 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, the current report 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 up-regulation of miR-32 in at least some human cancers including prostate carcinoma and multiple myeloma (25, 48) appears 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 appears to aid the phagocytic function of monocytes, which produces intracellular stress as a consequence of generation of ROS needed to dispose of phagocytised material. But since 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.

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## Abbreviations

<b>miRNA</b>	microRNA
<b>1,25D</b>	1,25-dihydroxyvitamin D <sub>3</sub>
<b>UTR</b>	untranslated region



<b>Bim</b>	Bcl-2-interacting mediator of the cell death, Bcl-2 like protein 11, Bim-EL
<b>Anti-miR-32</b>	antisense oligonucleotides to miR-32
<b>pre-miR-32</b>	precursor miR-32
<b>AraC</b>	arabinocytosine

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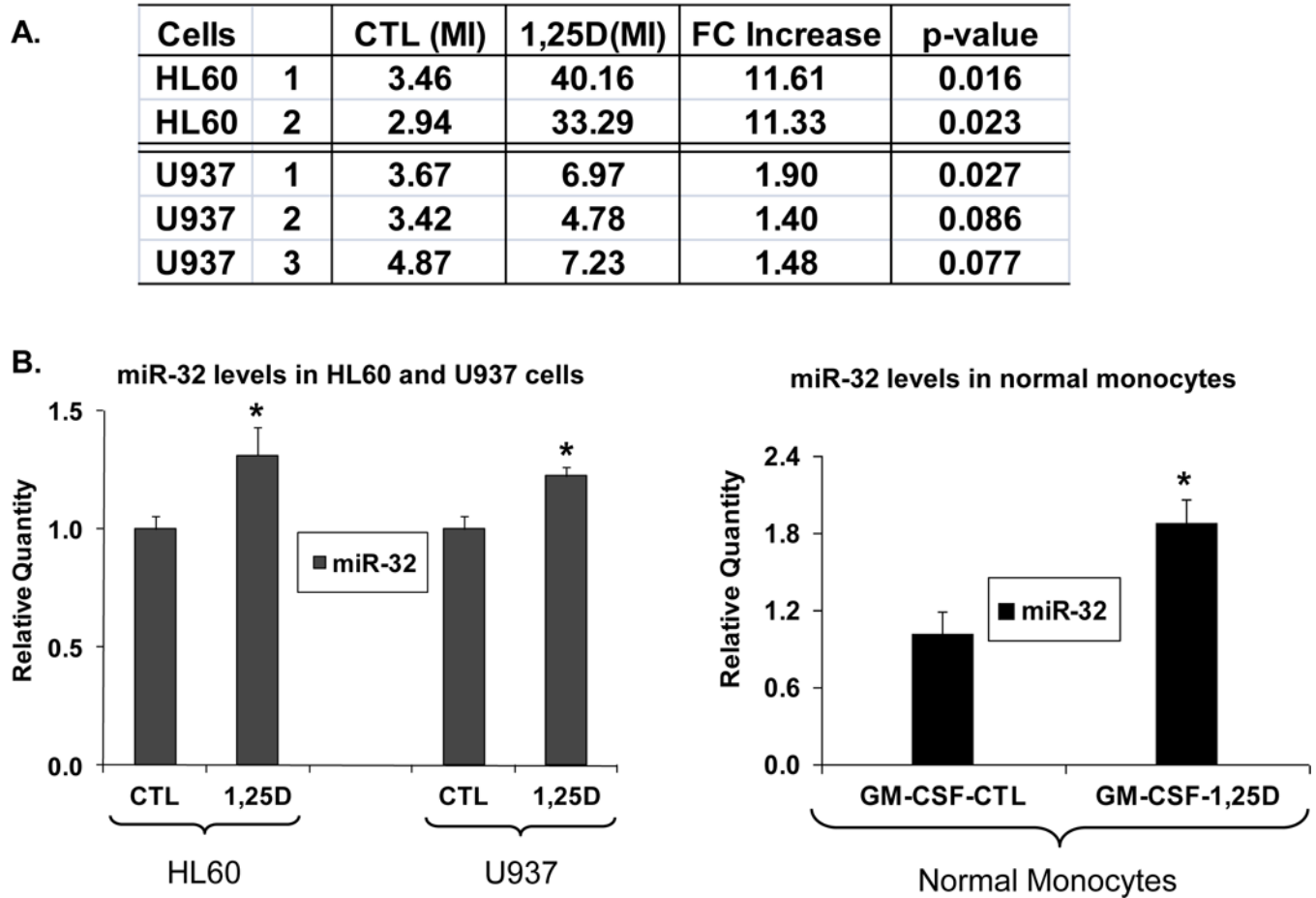
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## miR-32 is up-regulated by 1,25D.

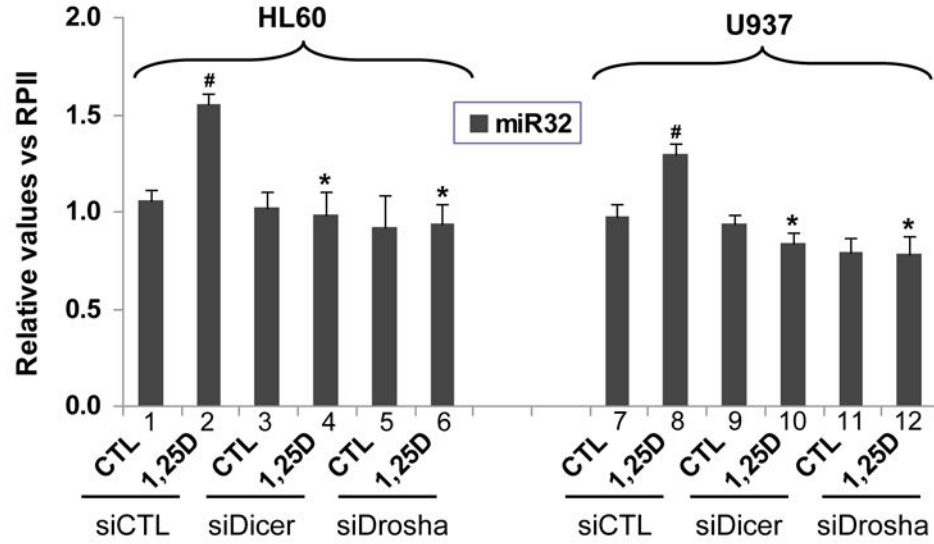


**Fig. 1. Expression levels of miR-32 are up-regulated by 1,25D in HL60 and U937 cells**  
 HL60 cells were exposed to 1 nM 1,25D for 48 h, and the less sensitive U937 cells to 10 nM 1,25D for 72 h, while normal monocytes to 100 nM 1,25D for 96 h. The miR-32 expression level was estimated using (A) miRNA microarray platform (24) and (B) TaqMan qRT-PCR assays. Asterisks show the statistically significant differences from the corresponding controls,  $p < 0.05$ ; bars represent mean values  $\pm$  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.

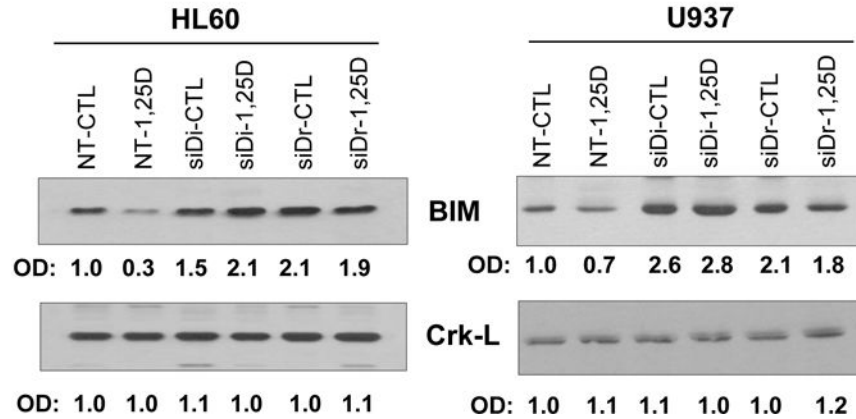


differences from the corresponding controls (CTL).  $p < 0.05$ ; bars represent mean values  $\pm$  SE,  $n = 4$ . The levels of Bim protein were determined by Western blotting and representative blots of four experiments are shown here with signal densities indicated below each panel. Calregulin was used as a loading control.

**A.** Transfection of siRNA against Dicer or Drosha results in decreased expression of miR-32.



**B.** Transfection of siRNA against Dicer or Drosha results in increased expression of Bim protein.

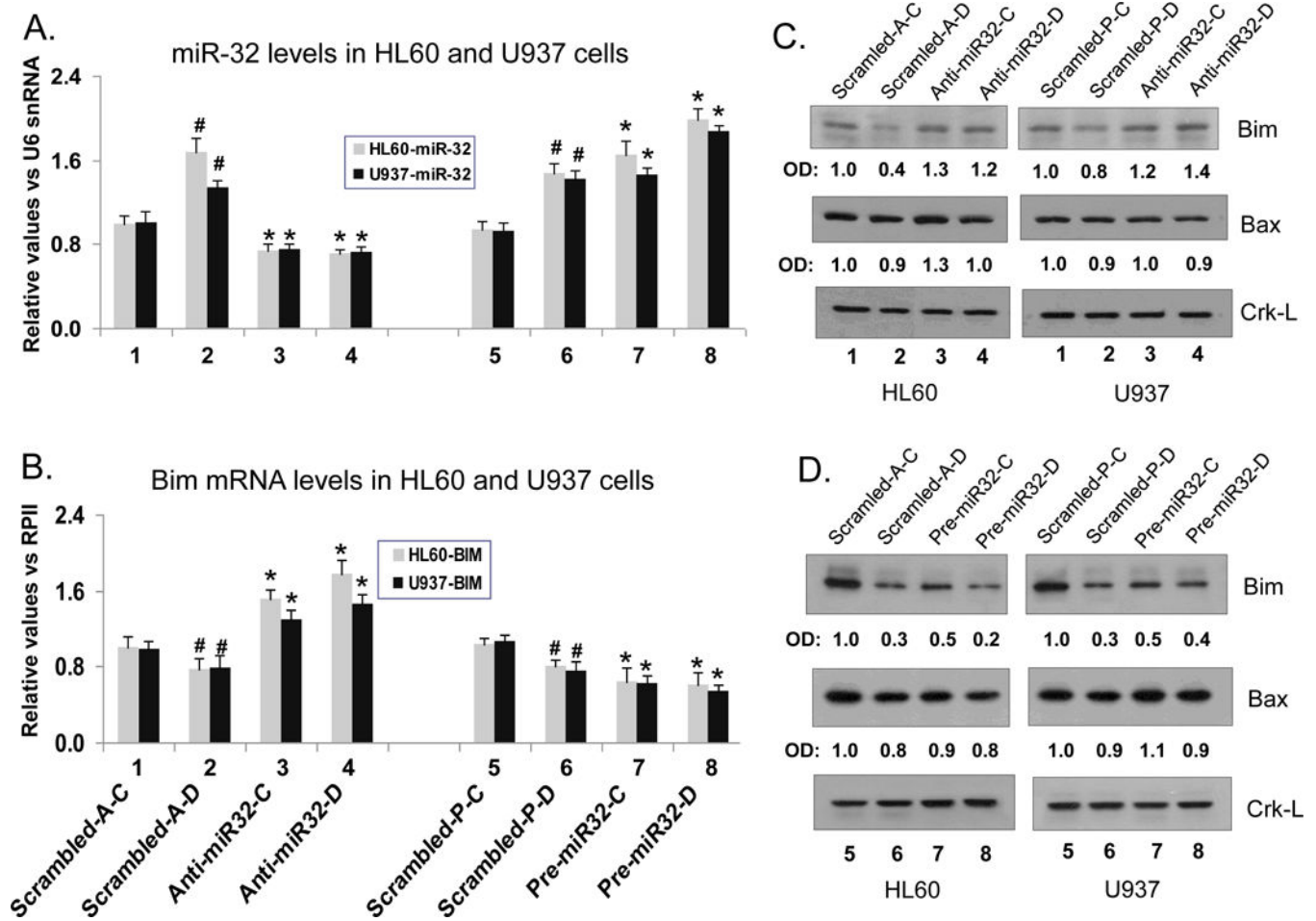


**Fig. 3. The expression of Bim protein is regulated by miRNA**

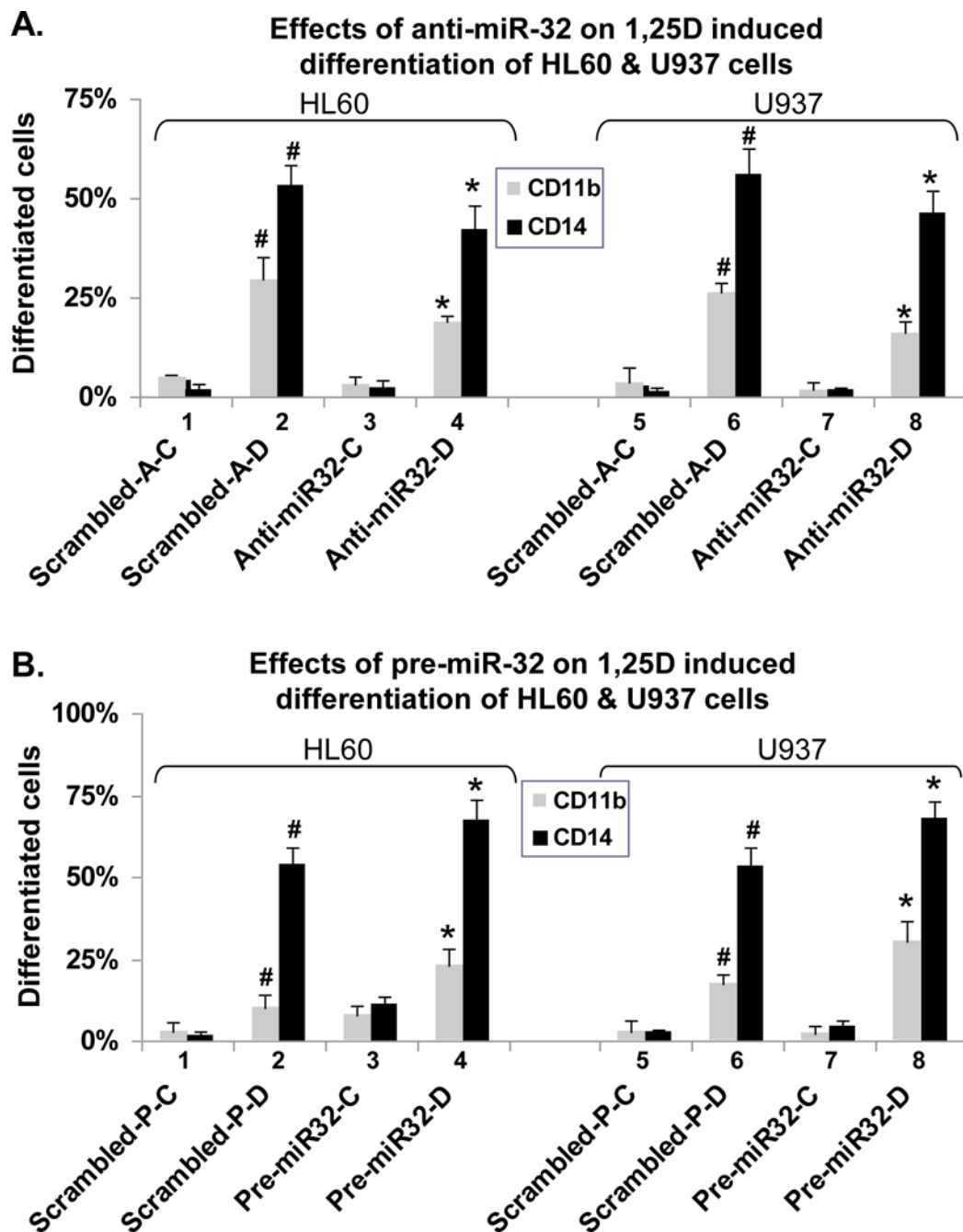
Transfection of siRNA against miRNA processing enzymes Drosha or Dicer results in: (A) abrogation of 1,25D-induced increase in the expression of miR-32, and (B) increased expression of Bim protein in HL60 and U937 cells. siRNA (20 nM) against Drosha or Dicer, or the non-targeting control (denoted siCTL in panels A, or NT-CTL in panels B) oligonucleotides were transfected into the cells for 48 h, then the cells were exposed to 1 nM (HL60) or 10 nM (U937) 1,25D for further 24 h. The level of miR-32 was determined by TaqMan qRT-PCR. The # symbol indicates the statistically significant differences in 1,25D-



treated cells (lanes 2 and 8) from the vehicle-treated cells (lanes 1 and 7). The asterisks (\*) show significant differences from the corresponding siCTL group, apparent only in 1,25D-treated groups.  $p < 0.05$ ; bars represent mean values  $\pm$  SE,  $n = 4$ . The levels of Bim protein and the loading control Crk-L were determined by Western blotting. Band densities are shown below each panel, which are representative blots of three experiments. NT, siCTL = Non-targeting siRNA control, siDi= siRNA against Dicer; siDr= siRNA against Drosha . The asterisks (\*) show significant differences from the corresponding siCTL group, apparent only in 1,25D-treated groups.  $p < 0.05$ ; bars represent mean values  $\pm$  SE,  $n = 4$ . The levels of Bim protein and the loading control Crk-L were determined by Western blotting. Band densities are shown below each panel, which are representative blots of four experiments. NT, siCTL = Non-targeting siRNA control, siDi= siRNA against Dicer; siDr= siRNA against Drosha.



**Fig. 4. Modulation of miR-32 expression by antisense oligonucleotides or by miR-32 precursor results in reciprocal changes in the expression of Bim mRNA and protein in AML cells** (A) HL60 and U937 cells were transfected with 20 nM antisense oligonucleotides against miR-32 (lanes 3 and 4) or 20 nM pre-miR-32 (lanes 7 and 8) for 48 h, then exposed to 1 nM and 10 nM 1,25D, respectively for further 48 h. MiR-32 levels were determined by TaqMan qRT-PCR. The # symbols indicate the statistically significant differences in 1,25D-treated cells (lane 2 or lane 6) from the corresponding vehicle-treated cells (lanes 1 and 5). The asterisks (\*) show the statistically significant differences from the corresponding (scrambled) controls transfected with non-targeting scrambled oligos (scrambled-A, lane 1 vs anti-miR-32 lane 3; or scrambled-P lane 5 vs pre-miR-32, lane 7). A comparison is also shown for 1,25D-treated cells lane 4 vs lane 2, and lane 8 vs lane 6).  $p < 0.05$ ; bars represent mean values  $\pm$  SE,  $n = 4$ . (B) Bim mRNA levels were determined by SYBRGreen qRT-PCR in the experiments described in (A) above. (C) Levels of Bim and Bax proteins after transfection of anti-miR-32, and (D) pre-miR-32, as determined by Western blotting. Crk-L was used as a loading and transfer control. OD ratio of each band versus CrkL is shown below each panel, which is representative of three experiments.



**Fig. 5. Modulation of miR-32 expression by antisense miR-32 and precursor miR-32 influences 1,25D-induced AML cell differentiation**

(A) Effects of anti-miR-32 on 1,25D induced differentiation of HL60 and U937 cells. (B) Effects of pre-miR-32 on 1,25D-induced differentiation. The conditions and symbols are described in the legend to Fig 4 and the determination of differentiation markers in “Materials and Methods”.

### Inhibition of miR-32 expression by antisense oligonucleotides increases the toxicity of AraC to HL60 and U937 cells

Table 1

HL60 and U937 cells were transfected with 20 nM antisense oligonucleotides against miR-32 for 48 h, and then exposed to 1 nM (HL60) or 10 nM (U937) 1,25D for further 48 h. Then the cells were exposed to 100  $\mu$ M AraC for further 24 h. To detect apoptosis cells were stained using 50  $\mu$ g/ml Annexin V and 20  $\mu$ g/ml propidium iodide 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  $\pm$  SE are shown, n = 4. C= vehicle control; D=1,25D-treated; A=anti-miR32; P=pre-miR32.

#### A: Anti-miR-32 increases toxicity of AraC to HL60 and U937 cells

Groups	Cells	Treatment	Viability Mean $\pm$ SD (%)	Early-Apts Mean $\pm$ SD (%)	Late-Apts Mean $\pm$ SD (%)	Total-Apts Mean $\pm$ SD (%)	SubG1 Mean $\pm$ SD (%)
1	HL60	Scrambled-A-C	79.3 $\pm$ 6.8	9.6 $\pm$ 2.9	22.3 $\pm$ 6.4	31.9 $\pm$ 6.5	9.9 $\pm$ 4.4
2	HL60	Scrambled-A-D	75.3 $\pm$ 10.5	7.6 $\pm$ 1.7	16.7 $\pm$ 7.7	24.3 $\pm$ 6.6	6.0 $\pm$ 2.1
3	HL60	Anti-miR32-C	<b>65.3 <math>\pm</math> 8.3</b>	9.7 $\pm$ 1.3	<b>31.9 <math>\pm</math> 7.9</b>	<b>41.6 <math>\pm</math> 6.5</b>	<b>16.6 <math>\pm</math> 4.7</b>
4	HL60	Anti-miR32-D	<b>63.0 <math>\pm</math> 10.3</b>	10.9 $\pm$ 2.4	<b>31.3 <math>\pm</math> 7.7</b>	<b>42.2 <math>\pm</math> 9.5</b>	<b>13.2 <math>\pm</math> 3.5</b>
5	U937	Scrambled-A-C	70.3 $\pm$ 5.0	10.4 $\pm$ 1.2	33.3 $\pm$ 6.6	43.7 $\pm$ 7.0	8.6 $\pm$ 1.5
6	U937	Scrambled-A-D	71.7 $\pm$ 4.2	9.5 $\pm$ 1.7	32.1 $\pm$ 3.6	41.6 $\pm$ 4.1	7.6 $\pm$ 3.1
7	U937	Anti-miR32-C	<b>61.0 <math>\pm</math> 5.5</b>	10.5 $\pm$ 2.1	<b>45.1 <math>\pm</math> 6.9</b>	<b>55.6 <math>\pm</math> 3.1</b>	<b>11.4 <math>\pm</math> 2.1</b>
8	U937	Anti-miR32-D	<b>63.3 <math>\pm</math> 3.8</b>	9.1 $\pm$ 2.4	<b>40.7 <math>\pm</math> 7.0</b>	<b>49.8 <math>\pm</math> 4.2</b>	<b>12.5 <math>\pm</math> 2.7</b>

#### B: Pre-miR-32 reduces toxicity of AraC to HL60 and U937 cells

Groups	Cells	Treatment	Viability Mean $\pm$ SD (%)	Early-Apts Mean $\pm$ SD (%)	Late-Apts Mean $\pm$ SD (%)	Total-Apts Mean $\pm$ SD (%)	SubG1 Mean $\pm$ SD (%)
1	HL60	Scrambled-P-C	77.0 $\pm$ 11.5	9.0 $\pm$ 2.2	27.3 $\pm$ 4.8	36.3 $\pm$ 6.7	8.4 $\pm$ 1.6
2	HL60	Scrambled-P-D	79.0 $\pm$ 10.5	7.0 $\pm$ 1.6	19.9 $\pm$ 4.9	26.9 $\pm$ 7.8	6.4 $\pm$ 1.8
3	HL60	Pre-miR32-C	79.5 $\pm$ 11.5	7.0 $\pm$ 1.2	<b>14.9 <math>\pm</math> 3.7</b>	<b>21.9 <math>\pm</math> 5.5</b>	<b>4.7 <math>\pm</math> 1.6</b>
4	HL60	Pre-miR32-D	80.5 $\pm$ 10.5	7.1 $\pm$ 2.1	<b>13.8 <math>\pm</math> 4.2</b>	<b>20.9 <math>\pm</math> 6.1</b>	<b>4.0 <math>\pm</math> 1.5</b>
5	U937	Scrambled-P-C	61.0 $\pm$ 5.1	15.3 $\pm$ 1.2	36.9 $\pm$ 6.3	52.2 $\pm$ 7.2	8.6 $\pm$ 1.9
6	U937	Scrambled-P-D	62.3 $\pm$ 7.2	12.5 $\pm$ 1.8	43.2 $\pm$ 6.0	55.7 $\pm$ 7.5	7.8 $\pm$ 2.3
7	U937	Pre-miR32-C	67.5 $\pm$ 6.0	<b>6.6 <math>\pm</math> 1.0</b>	35.5 $\pm$ 5.9	42.1 $\pm$ 7.1	5.3 $\pm$ 1.5
8	U937	Pre-miR32-D	68.0 $\pm$ 6.1	12.4 $\pm$ 1.9	<b>27.6 <math>\pm</math> 5.7</b>	<b>40.0 <math>\pm</math> 7.3</b>	<b>4.9 <math>\pm</math> 1.4</b>