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The *miR-17~92* cluster contributes to MLL leukemia through the repression of MEIS1 competitor PKNOX1

Yousaf A. Mian¹ and Nancy J. Zeleznik-Le^{1,2}

¹Molecular Biology Program, Loyola University Chicago, Maywood, IL 60153

²Department of Medicine, Loyola University Chicago, Maywood, IL 60153

Abstract

Mixed lineage leukemias have a relatively poor prognosis and arise as a result of translocations between the *MLL* (*KMT2A*) gene and one of multiple partner genes. Downstream targets of *MLL* are aberrantly upregulated and include the developmentally important *HOX* genes and *MEIS1*, as well as multiple microRNAs (miRNAs), including the *miR-17~92* cluster. Here we examined the contribution of specific miRNAs to MLL leukemias through knockdown studies utilizing custom anti-microRNA oligonucleotides. Combinatorial treatment against *miR-17-5p* and *miR-19a-3p* of the *miR-17~92* cluster dramatically reduces colony forming ability of MLL-fusion containing cell lines relative to non-MLL acute myeloid leukemia (AML) controls. To determine the mechanism by which these miRNAs contribute to leukemia, we validated *PKNOX1* as a target of both *miR-17-5p* and *miR-19a-3p*. *MEIS1* and *PKNOX1* are TALE domain proteins that participate in ternary complexes with *HOX* and *PBX* partners. Here we establish the competitive relationship between *PKNOX1* and *MEIS1* in *PBX*-containing complex formation and determine the antagonistic role of *PKNOX1* to leukemia in a murine *MLL-AF9* model. These data implicate the *miR-17~92* cluster as part of a regulatory mechanism necessary to maintain *MEIS1/HOXA9*-mediated transformation in MLL leukemia, indicating that targeting multiple non-homologous miRNAs may be utilized as a novel therapeutic regimen.

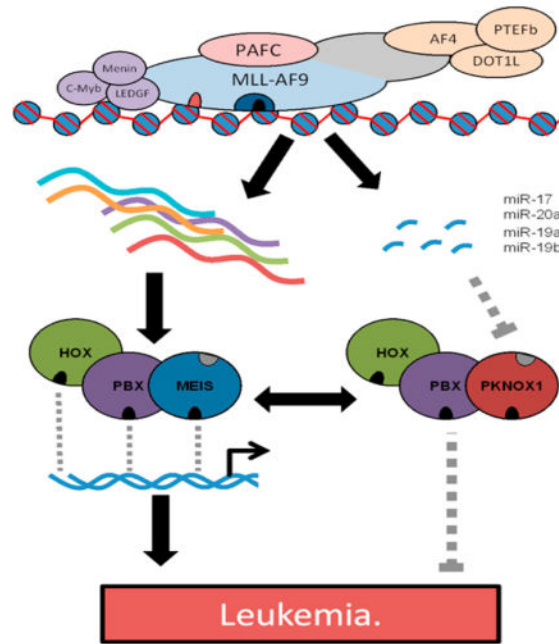
Graphical abstract

Corresponding author: Nancy J. Zeleznik-Le, Ph.D., Phone: 708-327-3368, Fax: 708-327-3342, nzelezn@luc.edu.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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Keywords

MLL; miRNA; *miR-17~92*; PKNOX1; MEIS1; leukemia

1. INTRODUCTION

Mixed lineage leukemias involving the *MLL* (*KMT2A*) gene account for about 5–10 percent of acute myeloid leukemias [1]. They arise as the result of chromosome translocations which produce fusion proteins expressing the amino-terminal portion of MLL and a carboxy-terminal fusion partner [2]. MLL fusion proteins inappropriately maintain the transcription of many target genes, including *HOX* genes [2, 3]. Recent studies have identified multiple miRNAs which are upregulated in MLL leukemias as compared to non-MLL AMLs [4].

MiRNAs are small non-coding RNAs (~22 nt) that post-transcriptionally downregulate the expression of mRNA target genes (reviewed in [5, 6]). Studies by our lab and others have identified several miRNAs upregulated in MLL leukemia including *miR-196b* and the *miR-17~92* cluster [7–9]. The *miR-17~92* cluster (also known as *oncomiR-1*) consists of 6 miRNAs co-expressed from a single transcript (*miR-17-5p*, *miR-18a-5p*, *miR-19a-3p*, *miR-20a-5p*, *miR-19b-3p-1*, and *miR-92a-3p-1*) [10] and is directly regulated by MLL [9]. Within this cluster, several miRNAs are highly related in sequence and form miRNA families. Family designation and target mRNA recognition is primarily mediated by nucleotides 2–7 of the miRNA, commonly referred to as the ‘seed region’ [11]. *miR-17-5p* and *miR-20a-5p* are highly homologous and share the same seed sequence (reviewed in [12]). Similarly, *miR-19a-3p* and *miR-19b-3p-1* are also highly homologous with identical seed regions and form a miRNA family (reviewed in [12]). The *miR-17~92* cluster targets genes involved in processes including cell death, cell cycle, development, and hypoxia

(extensively reviewed in [13, 14]). Validated targets for the *miR-17~92* cluster in various cancer models include *PTEN*, *AML1* and *E2F1* [15–17]. Intriguingly, several mRNAs have been validated as targets of more than one miRNA from the *miR-17~92* cluster, suggesting that miRNAs may act cooperatively to regulate targets. For example, *PTEN* has been validated as a target for both *miR-17-5p* and *miR-19a-3p* [17, 18].

Enforced overexpression of *miR-17-5p~19b* increases the colony forming ability of MLL-ELL (*KMT2A-ELL*) transformed bone marrow cells [9]. Overexpression of the *miR-17~92* cluster results in downregulation of 363 potential target genes, suggesting that dysregulation of the *miR-17~92* cluster has broad implications in target mRNA regulation [9]. Within MLL leukemia, *miR-17-5p* regulates *CDKN1A* (*p21*) expression [19]. Overexpression of *miR-17~19b* accelerated the onset of MLL-AF10 leukemia in a murine model, which was phenocopied by shRNA knockdown of *CDKN1A* (*p21*) [19]. The growing number of validated targets for each miRNA suggests that miRNAs are not limited to single targets, and that other, as yet non-validated targets, also likely contribute to the disease process. However, the mechanisms by which the *miR-17~92* cluster contribute to MLL leukemia are not yet fully defined.

HOX genes are the most well studied MLL targets, encoding transcription factors critical to body patterning, development, and hematopoiesis (reviewed in [20]). Enforced expression of *Hoxa9* and *Meis1* in murine bone marrow progenitor cells is sufficient to induce AML [21]. Previous studies have shown *HOXA9* is essential to MLL leukemias [22, 23]. *HOX* proteins function in a ternary complex containing PBX and MEIS1 partners. [24, 25]. Both the binary and ternary complex forms function to regulate target gene transcription [26]. Deletion of the Pbx-interacting domains of Meis1 abrogated its leukemogenic potential when co-infected with a *Hoxa9* expressing virus [27]. PBX3 is upregulated in MLL leukemia and disruption of the *HOX*-PBX interaction through a small peptide inhibitor promoted apoptosis and decreased expression of known *HOX*-MEIS targets in an MLL leukemia cell line [28]. These findings suggest that *HOX*-MEIS activity requires PBX participation.

Several technologies have emerged to target miRNAs utilizing chemically modified oligonucleotides complementary to the miRNAs (reviewed in [34]). Consisting of modifications to the phosphodiester bonds and the ribose backbone, antagomirs have increased target affinity and RNA stability relative to RNA:RNA duplexes [35, 36]. In this study, we target miRNAs both individually and in combination as a potential therapeutic approach for MLL leukemia. We find that use of a combination of antagomirs functions as a potent anti-leukemia treatment *in vitro*. Furthermore, we validate *PKNOX1* as a target of *miR-17~92* cluster miRNAs, which regulates the composition of functionally distinct PBX-containing complexes. This study establishes one mechanism by which miRNA inhibition disrupts the leukemogenic potential of MLL leukemia.

2. MATERIALS AND METHODS

2.1. Retroviral constructs and infections

MIGR1 [37], *MSCV-MLL-AF9* [37], and *MSCV-pKOF2-Prep1-EGFP* [32] retroviral constructs were previously described. Bone marrow isolation, enrichment, and infection for

generation of *MLL-AF9* transformed bone marrow was performed as previously described [7]. Additional infection with *MIGR1* or *pKOF2-Prep1-EGFP* was performed on *MLL-AF9* transformed bone marrow cells that had been serially replated in methylcellulose for 4 weeks. *pRL-TK* (Promega, Madison, #E2241), *MSCV-PIG* [10] and *MSCV-PIG miR-17-5p-19b* [10] have been described previously. Luciferase constructs were generated by cloning the 3' UTR of *PKNOX1* from human genomic DNA into the *pmiR-Report* plasmid (ThermoFisher, Waltham, #AM5795). Mutations to the miRNA binding sites were generated using the Stratagene Multi-site directed mutagenesis kit (Stratagene, La Jolla, California #200514). Construct details available upon request.

2.2. Cell culture and transfection

MOLM-13, MV4-11 and RS4;11 (DSMZ, Germany) and HEK293T cells were cultured using standard conditions. HEK293T cells were transfected using CalPhos Mammalian transfection system per manufacturer's instructions (ClonTech, Mountain View, #631312). For experiments examining the role of *Pknox1* in *MLL-AF9* transformed murine bone marrow, cells were generated as previously described [7]. After serial replating in methylcellulose for 4 weeks, cells were acclimated to liquid culture for 1 week and subjected to additional spinoculations with either *MIGR1* or *MSCV-pKOF2-Prep1-GFP*.

2.3. Antagomir modifications and treatment

Antagomirs were synthesized by Dharmacon (Lafayette, Colorado) with modifications described by Krutzfeldt [35, 36] All oligos were complementary to targeted miRNAs and followed a uniform pattern of modifications, including: 2'-O-Me modification at all nucleotides, and replacement of first 2 and last 4 phosphodiester linkages with phosphorothioate linkages. All oligos possess a cholesterol group appended to the 3' end (depicted in Supplementary Table 1). Fluorescent labeled antagomirs contained an additional DY547 fluorescent label appended to the 5' end. Antagomirs were added to media at the final concentrations indicated in figure legends.

2.4. RNA isolation and real-time polymerase chain reaction

RNA was extracted according to manufacturer's protocol using TRI Reagent (Sigma, St. Louis, #T9424) from cells treated with antagomirs for 72 hrs in liquid culture. cDNA synthesis was performed using 10 ng of total RNA using Applied Biosystems miRNA Reverse Transcription kit (Life Technologies, Carlsbad, #4366596). Real-Time PCR was performed on an Applied Biosystems 7300 cycler using Applied Biosystems Taqman assays for *hsa-miR-17-5p*, *hsa-miR-19a-3p*, or *RNU6*. Data was analyzed using the Delta Delta Ct method [38].

2.5. Methylcellulose colony assays

Methylcellulose colony assays for human cell lines were performed using H4100 Methocult methylcellulose (Stemcell, Vancouver, #04100) supplemented with a final serum concentration of 10% FBS. Cells were analyzed and collected after 7–8 days in culture. Colonies were imaged under an Olympus microscope. Murine cells were plated in H3234 Methocult supplemented with IL-3 (10 ng/mL), IL-6 (10 ng/mL), and SCF (100 ng/mL).

2.6. Cell proliferation Assay

Viable cell number for human cell lines was assessed after 4 days of antagomir treatment using Promega Cell-Titer-Glo kit (Promega, Madison, #G7570) according to manufacturer's instructions. Viable cell number for *MLL-AF9* transformed murine bone marrow was performed using cell titer glow at days 3, 5, and 7. Luminescence was measured on a Glomax 96 microplate luminometer. Remaining cells were collected for cell cycle analysis. Cells were fixed and stained with propidium iodide and analyzed via FACS. All FACS data was analyzed using Watson Pragmatic function within Flowjo Software Package (Version 9.03).

2.7. Fluorescence Activated Cell Sorting (FACS)

Cellular uptake of antagomirs was detected by FACS analysis. Live cells were treated with either a control or a fluorescent-human labeled antagomir. Mean fluorescence intensity was determined in FlowJo. Cell surface marker staining was performed as previously described [37] using anti-CD117-APC (eBioscience, San Diego, # 17-1171-82), anti-CD11B-PE (eBioscience, San Diego, #12-0112-85), anti-GR-1-PE-Cy5 (eBioscience, San Diego, #15-5931-81). Data was processed using FlowJo Software Package.

2.8. Luciferase Assays

HEK293T cells were plated at 6.0×10^5 cells well in 24 well plates and co-transfected with 120 ng of pmiR-Report reporter construct (wild-type or mutant 3'UTR for *PKNOXI*), 10 ng of *pRL-TK*, and 600 ng of either *MSCV-PIG* or *MSCV-PIG-miR-17~19b*. Cells were cultured for 48 hrs and collected for luciferase analysis with the Promega Dual-Luciferase Reporter Assay System (Promega, Madison, #E1910) according to manufacturer's instructions.

2.9. Co-Immunoprecipitation (co-IP) and Western Blotting

Co-IP was performed in IPH buffer using Agarose A beads (Santa Cruz Biotechnology, SC-2001). All equilibration and washes were performed with IPH buffer (50 mM Tris-HCl, 150 mM NaCl, 5mM EDTA, 0.5% NP-40) with freshly added protease inhibitor cocktail (Sigma Aldrich, St. Louis, #P8340).

Proteins were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes using Trans BlotTurbo transfer system (BioRad, Hercules, CA). Membranes were probed with anti-PREP1 (N15) (Santa Cruz Biotechnology, Santa Cruz, #SC-6245), anti-Pbx1/2/3 (C-20) (Santa Cruz Biotechnology, Santa Cruz# SC-888), anti-Meis1 (Millipore, Billerica, #05-779), anti-Hoxa9 (Millipore, Billerica, #07-178), or anti-beta-Actin (Sigma, St. Louis, #A5441). Secondary antibody probing was performed with TrueBlot anti-rabbit (eBioscience, San Diego, #18-8816-31), anti-mouse-HRP (GE Life sciences, Piscataway, #NA931VS), or anti-Rabbit-HRP (GE Life sciences, Piscataway, #NA934VS).

3. RESULTS AND DISCUSSION

3.1. Antagomir uptake occurs in a dose-dependent manner

Previous studies by our lab and others have utilized antagomirs to examine the role of specific miRNAs in a murine model of MLL leukemia [7, 39]. To determine the viability of antagomirs as a potential novel therapeutic, antagomir uptake in human MLL leukemia cell lines was examined by FACS analysis of a fluorescent labeled antagomir (Figure 1A,B). A linear correlation of antagomir uptake was found with dosage up to 2 μ M of antagomirs across multiple MLL fusion possessing leukemia cell lines. Knockdown was assessed at 2 μ M for MOLM-13 and MV4-11 cells and 1 μ M for RS4;11 cells utilizing antagomirs against *miR-17-5p* or *miR-19a-3p* (Figure 1C,D). Specific antagomir treatment resulted in significant knockdown of the targeted miRNA relative to cells treated with either a control antagomir or vehicle (Figure 1C,D). Collectively, these results indicate that antagomir treatment of human cell lines results in a dose dependent uptake with effective and specific knockdown of targeted miRNAs.

3.2. Combined inhibition of *miRs-17-5p* and *20a-5p* or *miRs-17-5p* and *19a-3p* reduces colony formation and increases differentiation of MLL leukemia cells

We hypothesized that increased expression of individual miRNAs is essential for maintaining MLL leukemia cell proliferation and clonogenic capacity. MLL leukemia cell lines were treated with antagomirs directed against a subset of miRNAs overexpressed in MLL leukemia to examine the functional requirement of specific miRNAs in MLL leukemia. We utilized cell lines with *MLL-AF9 (KMT2A-MLLT3)* (MOLM-13) or *MLL-AF4 (KMT2A-AFF1)* (MV4-11, RS4;11) translocations. Treatment with *anti-miR-20a-5p* produced the greatest decrease in colony forming ability in all cell lines, while treatment with either *anti-miR-17-5p* or *anti-miR-20a-5p* was sufficient to completely block colony formation in MV4-11 (Figure 2A). Antagomirs against *miR-19a-3p* caused no significant decrease relative to treatment with a control antagomir when used as a single antagomir treatment (Figure 2A). *Anti-miR-19b-3p-1* treatment caused a decrease relative to both vehicle and control antagomir treatments in MV4-11 cells alone (Figure 2A).

Combinatorial antagomir treatments on human cell lines were examined to determine whether simultaneously targeting multiple miRNAs with the same seed sequence or targeting multiple miRNAs with different seed sequences would potentiate the effects on colony forming capacity or proliferation (Figure 2). Treatment with *anti-miR-17-5p* plus *anti-miR-19a-3p* produced a dramatic effect beyond that observed for either of these antagomirs individually (Figure 2A). This combination produced an effect beyond an additive effect in MOLM-13. We further examined colony and cell morphology for combinatorial treatments (Figure 2B). Morphologically, cells appear more differentiated with a greater cytoplasmic:nuclear ratio and increased vacuolarity. Combinatorial treatment against *miR-17-5p*, and *miR-19a-3p* resulted in a change in cell morphology in all MLL leukemia cell lines, but not in non-MLL AML cell lines (Figure 2B). Colonies were more sparse and contained fewer cells after treatment with either *anti-miR-17-5p* and *20a-5p*, or *anti-miR-19a-3p* and *19b-3p-1*, or *anti-miR-17-5p* and *19a-3p* (Figure 2A,B). Further, a decrease in cell number that was greater than the decrease in colony number in the

combinatorial treatments was observed, suggesting that in addition to inhibition of colony forming ability, antagomir treatments either inhibited proliferation, or increased cell death and/or differentiation.

We have determined the requirement of specific miRNAs in the *miR17~92* cluster to maintain the transformed phenotype of MLL leukemia by antagonizing miRNAs over-expressed in this subtype of leukemia. In particular, antagonizing both *miR-17-5p* and *miR-20a-5p*, or both *miR-17-5p* and *miR-19a-3p* had the most dramatic effects (Figure 2A, B). Targeting related miRNAs of the same family could potentially circumvent redundant functions caused by multiple miRNA family members' ability to target the same mRNA transcript. Antagonizing unrelated miRNA family members shows a more dramatic effect beyond that observed for targeting only individual miRNA family members, suggesting that the miRNAs from different families also function cooperatively. This could be due to regulation of different mRNAs or because multiple miRNAs simultaneously and more efficiently target the same critical mRNA.

3.3. miRNA inhibition causes a modest decrease in cell number and increased G1/G0 cell cycle accumulation

Human cell lines were treated with antagomirs in liquid culture to examine proliferative defects and separate these effects from colony forming ability. Both individual treatments and combinatorial treatments were simultaneously tested in human MLL leukemia cell lines (Figure 3). Although individual treatments were much less dramatic, decreases largely mirrored the colony assay data (Figure 3A). Treatment with *anti-miR-17-5p* or *anti-miR-20a-5p* resulted in a decrease in total cell number at 4 days, while treatment with *anti-miR-19a-3p* or *anti-miR-19b-3p-1* had no individual effect. Combinatorial treatment against *miR-17-5p* and *20a-5p* resulted in a decrease in total cell number for RS4;11 cells, while *anti-miR-17-5p* and *20a-5p* and *anti-miR-17-5p* and *19a* both had significant effects in MOLM-13 cells. Surprisingly, we did not detect any decrease in cell number relative to control in MV4-11 cells. Antagomir-treatment produced a less dramatic effect in liquid culture than in colony assays. To further examine effects on proliferation, cell cycle was examined by propidium iodide staining. Decreased cell numbers were accompanied by a modest accumulation of cells at G0/G1 (Figure 3B). These data indicate that decrease in colony forming ability was accompanied by a mild proliferative defect.

3.4. PKNOX1 is a valid target of the *miR-17~92* cluster

We next sought to determine potential mRNA targets relevant to MLL leukemia, focusing on common putative targets of both the *miR-17* and *miR-19* families with functions related to colony forming ability. While shRNA knockdown of *CDKN1A* (*p21*) in an MLL leukemia model phenocopies overexpression of the *miR-17-5p~19b*, it is not currently known to be targeted by *miR-19a-3p*. Our antagomir treatment data indicate a more severe phenotype upon combinatorial treatment with *anti-miR-17-5p* and *anti-miR-19a-3p*. As a result, we focused on a target that is regulated by both *miR-17-5p* and *miR-19a-3p*. Search results using multiple target prediction algorithms (miRanda, TargetScan, Diana-mT) identified the mRNA for the TALE domain protein PKNOX1 as an intriguing possible target (not shown). Our search identified additional common targets of the *miR-17* and *miR-19* families. These

included targets that have been validated in the literature, such as *PTEN* [17, 40] and targets that have yet to be experimentally validated, such as *CASP8*. However, PKNOX1 was intriguing for both the strength of the predicted miRNA:mRNA interactions and the previously documented functional roles of PKNOX1. MiRNA targeting algorithms predict that the *miR-17~92* cluster targets the 3'UTR of the *PKNOX1* mRNA at multiple sites. *miR-17-5p* and *miR-20a-5p* putatively target the *PKNOX1* 3'UTR at 2 distinct sites, while *miR-19a-3p* and *19b* putatively target the *PKNOX1* 3' UTR at a single site (Figure 4A,B). All sites are evolutionarily conserved between mouse and human.

PKNOX1 (also known as PREP1) is a homolog of MEIS1 and, along with PKNOX2 and MEIS1/2/3/4 proteins, forms the MEINOX family [29]. PKNOX1 was initially characterized as a PBX binding partner with an important role in embryonic development, whose loss results in embryonic lethality through multiple defects [41]. *Pknox1* has been further characterized as a tumor suppressor in an Eμ-Myc model of lymphoma [42]. PKNOX1 participates in HOX-PBX protein complexes, utilizing the same interaction domains as MEIS1 [30]. PKNOX1 and MEIS1 are highly homologous in the domains required for both PBX interaction and DNA binding and interact with the same surface on PBX partners [31]. However, the oncogenic activity of MEIS1 is located in the C-terminal domain, which differs from that of PKNOX1 [27, 32, 33]. *Meis1* cooperates with *Hoxa9* in leukemic transformation; whereas co-expression of *Pknox1* with *Hoxa9* lacks this effect [32]. However, chimeric Pknox1 proteins which append the Meis1 C-terminus to the Pknox1 protein can restore acceleration of leukemia development [32]. Studies of *Pknox1* and *Meis1* in murine development indicate that Pknox1 and Meis1 largely regulate distinct sets of genes, but often have opposing functions where their targets overlap [26].

To determine the function of predicted *miR-17~92* cluster targets in the *PKNOX1* 3'UTR, we performed luciferase reporter assays. Mutations were generated for the putative miRNA response elements (MREs) targeted by the *miR-17* family, the *miR-19* family, or both (Figure 4B). Mutation of either MRE individually or of all *miR-17~92* cluster-targeted MREs resulted in an increase in luciferase expression relative to a wild-type reporter construct, indicating a valid regulatory relationship between the miRNAs and the *PKNOX1* mRNA (Figure 4C). We determined steady-state levels of PKNOX1 in MLL leukemia cell lines along with the non-MLL K562 cells (Figure 4D). PKNOX1 levels were markedly lower in MLL leukemia cell lines relative to K562 or HEK293T cells, supporting our findings that PKNOX1 is downregulated in MLL leukemias (Figure 4D).

Our data demonstrate that *PKNOX1* is a valid target of the *miR-17~92* cluster. Multiple miRNAs from this cluster regulate *PKNOX1* mRNA through binding to separate miRNA response elements. The redundancy of miRNAs targeting the same mRNA suggest that the relationship of *miR-17~92* and *PKNOX1* functions as an important regulatory switch which may respond to various cellular signals. While binding affinity for the *PKNOX1* transcript is predicted to be thermodynamically stable, it is likely that other mRNAs are targeted simultaneously dependent on availability of targets.

3.5. PKNOX1 and MEIS1 compete for participation in PBX-HOX complexes

To test whether miRNA levels resulted in a change in PKNOX1 protein level, *miR-17~19b* was expressed in HEK293T cells, which express easily detectable PKNOX1, and endogenous PKNOX1 protein was determined by western blot. Increasing concentrations of *miR-17~19b* resulted in a decrease in PKNOX1 protein levels (Figure 5A). To determine whether antagomir treatment also mediated PKNOX1 dysregulation, PKNOX1 levels were determined after combinatorial antagomir treatment (Figure 5B). PKNOX1 levels increased in response to treatment with antagomir combinations against either the *miR-17* family, the *miR-19* family, or against both *miR-17-5p* and *miR-19a-3p* relative to a control antagomir treatment (Figure 5B). These results further confirm that *miR-17~92* cluster miRNAs regulate *PKNOX1*.

PKNOX1 and MEIS1 share high homology in their PBX-interacting domains and their homeodomains. Thus, we hypothesized that increased MEIS1 expression would compete with PKNOX1 for PBX binding, thus causing decreased PKNOX1 inclusion in these complexes. To test the competitive relationship of PKNOX1 and MEIS1, HEK293T cells were transfected with increasing amounts of a *Meis1*-expressing plasmid and co-immunoprecipitated for PBX-containing complexes using an antibody that recognizes all PBX family proteins. With increasing levels of Meis1 protein, less PKNOX1 was present in PBX complexes, indicating a competitive relationship (Figure 5C).

Recent studies by the Blasi group support and complement our findings [43, 44]. They found that Prep1 (*Pknox1*) competes with Meis1 for binding to Pbx1, and *Prep1* depletion enabled *Meis1* to transform MEFs [43]. Additionally, depletion of *Prep1* accelerated the onset of *Meis1-Hoxa9*-mediated leukemogenesis in a murine model [44]. They hypothesized that increasing PREP1 levels may be an important area for future therapeutic development [43]. Studies performed by the Chen lab indicate that *HOXA9* and *MEIS1* are negatively regulated by *miR-196b*, one of the miRNAs overexpressed in MLL leukemia [45]. Although this causes an initial delay in the onset of leukemia, increased *miR-196b* expression ultimately contributes to a more aggressive leukemia through repression of the tumor suppressor FAS [45]. This effect could thus limit the availability of HOXA9 and MEIS1 for the formation of HOX-PBX-MEIS complexes. HOX-PBX3 protein-protein interaction is also essential for MLL leukemia development and inhibition of this interaction with a small peptide selectively kills cells dependent on *HOXA* and *PBX3* overexpression [28]. Collectively, these findings implicate ternary HOX-PBX-MEIS complexes in MLL leukemia development and support a model of competition between PKNOX1 and MEIS1 for inclusion into HOXA9-PBX3 containing complexes in MLL leukemia.

3.6. Enforced *Pknox1* expression decreases the colony formation ability, promotes differentiation, and results in a decrease in cell number of MLL-AF9-transformed cells

Pknox1 lacks the oncogenic potential of *Meis1* in a *Hoxa9*-dependent murine transformation model [32]. However, the role of *Pknox1* in the context of MLL leukemia remains to be tested. We hypothesized that *Pknox1* would have an inhibitory role in MLL leukemia. MLL-AF9 transformed murine bone marrow cells were infected with a *Pknox1*-expressing virus or a control virus. Enforced expression of *Pknox1* caused a decrease in colony number relative

to a control vector-infected cells (Figure 6A). The decrease in colony number was primarily caused by a decrease in Type1 colonies which represent the least differentiated colony type (Figure 6B, D). Total cell number after one week in colony assays was also decreased in *Pknox1*-expressing cells relative to cells infected with a control virus (Figure 6C), mirroring the effects observed in *anti-miR-17-5p* and *miR-19a-3p* combinatorial antagomir treatments. Enforced overexpression of *Pknox1* resulted in both a decrease in CD117 expression and an increase in Mac-1 and Gr-1 myeloid differentiation markers, supporting the colony phenotype data (Figure 6E). The effects of increased *Pknox1* expression on proliferation of *MLL-AF9*-transformed bone marrow cells was also examined in liquid culture. Enforced *Pknox1* expression inhibited the proliferation of *MLL-AF9*-transformed cells (Figure 6F). As with the human leukemia cell experiments, a modest accumulation of cells at G0/G1 was observed (Figure 6G). This observation is consistent with the effects of antagomir treatments on proliferation and colony-forming capacity of MLL leukemia cell lines.

Murine studies found that infection with *Pknox1*-expressing virus fails to accelerate leukemia similarly to *Meis1* when co-expressed with *Hoxa9* [32]. Our experiments demonstrate that exogenous over-expression of *Pknox1* negatively affects MLL leukemia cell proliferation and colony-forming ability. We found that *Pknox1* overexpression in a murine MLL leukemia model decreased colony forming ability, primarily from a loss of less differentiated colonies. While human MLL cell lines do not form compact colonies like *MLL-AF9* transformed murine bone marrow, the ability to form colonies is restricted to a subset of cells. Colony forming capability has been used as a surrogate of leukemia stem/progenitor cell activity in the MLL leukemia model system. Thus, loss of colony forming ability in response to antagomir treatments, without a more dramatic decrease in liquid culture growth, suggests that MLL leukemia stem/progenitor cell activity may be predominantly targeted by these miRNAs. Future work could determine whether specific cell contact-mediated pathways are particularly influenced by *miR-17-92* cluster miRNAs in MLL leukemia.

4. CONCLUSIONS

Complex regulatory circuits involving miRNAs are starting to be defined in MLL leukemia. This includes regulation of HOX-PBX-MEIS complexes. Both *HOXA9* and *MEIS1* are directly regulated by MLL and are necessary for MLL leukemia development [3, 22, 44, 46] MiRNAs in the *miR-17-92* cluster are also overexpressed in MLL leukemia. Here we show that *miR-17-5p* and *miR-19a-3p* of the *miR-17-92* cluster target *PKNOX1* and that antagonizing these miRNAs causes increased PKNOX1 expression. We further demonstrate that MEIS1 and PKNOX1 compete for binding in HOX-PBX containing complexes and that manipulation of relative MEIS1/PKNOX1 levels alters complex composition. Within *MLL-AF9* transformed bone marrow, exogenous overexpression of *Pknox1* has a detrimental effect of colony forming ability and results in an increase in differentiation markers. Together, this suggests that HOX-PBX complexes are a limiting factor in MLL leukemia and are amenable to functional disruption through antagonizing critical regulatory miRNAs. We conclude that expression of several miRNAs of the *miR-17-92* cluster are critical for the continued survival of MLL leukemia. Some of this effect is likely mediated through

regulation of *PKNOXI*, indicating this pathway would be worthwhile to explore as potential therapeutic target for MLL leukemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Treatment with anti-miRNA oligos decreases MLL leukemia cell line colony formation.
- The *miR-17* and *miR-19* miRNA families target the MEINOX family member *PKNOX1*.
- PKNOX1 and MEIS1 compete for inclusion into PBX-containing complexes.
- Enforced *Pknox1* expression reduces MLL-AF9 leukemia colony formation ability.

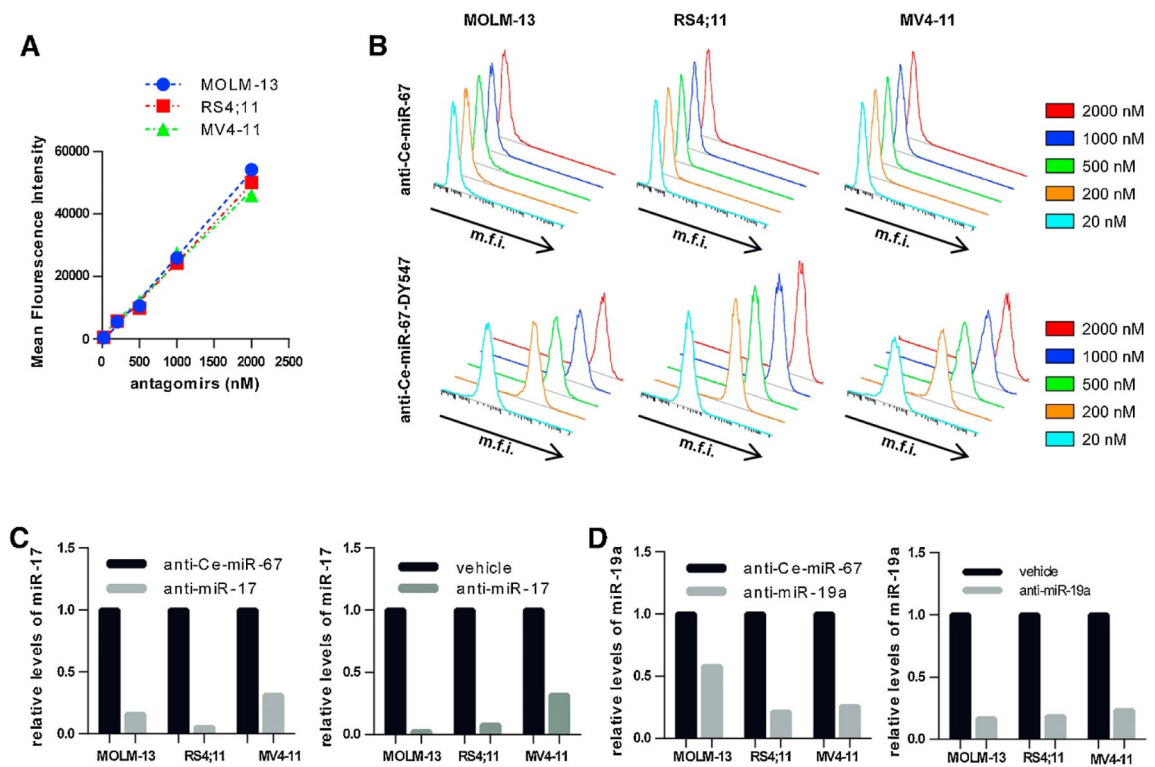


Figure 1. Antagomir treatment occurs in a dose dependent manner and results in a knockdown of target miRNA

A–B) Antagomir uptake was determined by FACS analysis using a fluorescently labeled antagomir at multiple concentrations (20 nM–2000nM). A) Mean fluorescence intensity of cells was plotted at antagomir treatment concentrations of 20 nM, 200 nM, 500 nM, 1000 nM, and 2000 nM. B) Example plots of uptake for MOLM-13, RS4;11 and MV4-11 treated with increasing concentrations for a control antagomir (top) and its fluorescent-labelled counterpart (bottom). C–D) Antagomir-mediated knockdown of target miRNAs was verified at final treatment concentration in liquid culture. C) Knockdown shown for *miR-17-5p* levels relative to a control antagomir (left) or a vehicle control (right). D) Knockdown shown for *miR-19a-3p* levels relative to a control antagomir (left) or a vehicle control (right). *Anti-Ce-miR-67* was used as a control to assess knockdown in all cell lines.

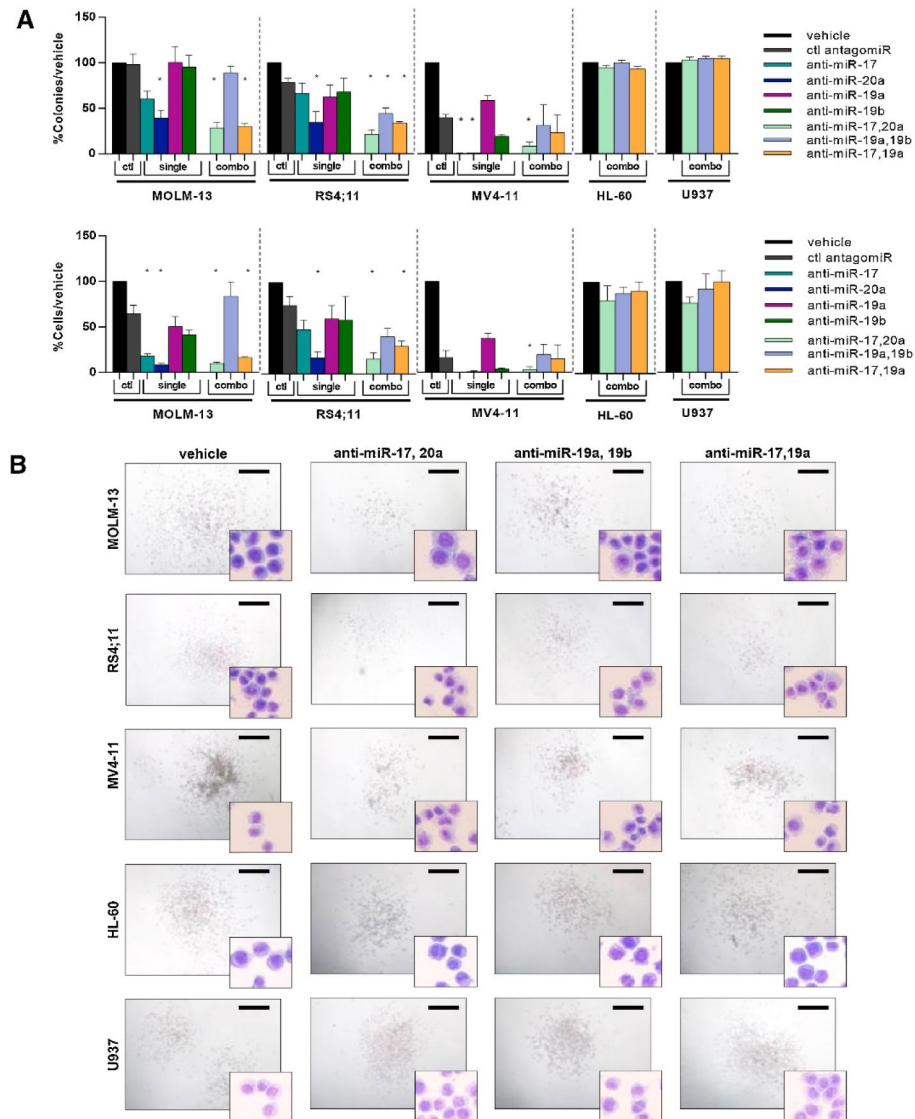


Figure 2. Antagomir treatment results in a decrease in colony forming ability

A–B) Antagomir treatments were performed against individual miRNAs and combinations of miRNAs at a total concentration of 2 μ M for MOLM-13 and MV4-11 and 1 μ M for RS4;11. MOLM-13 and RS4;11 were tested in three independent experiments with each experiment performed using duplicate plates. MV4-11 was tested in two independent experiments with each experiment performed using duplicate plates. Significance was tested against control antagomirs ($*$ = $p < 0.05$). *Anti-Ce-miR-67* was used as a control for RS4;11, and *anti-miR-10a* was used as a control for MOLM-13 and MV4-11. HL-60, and U937 were tested in three independent experiments with each experiment performed using duplicate plates. E) Antagomir treated colonies were photographed and collected for Wright-Giemsa staining. Representative slides shown for both colony and cellular morphology. Scale bars represent 200 μ m.

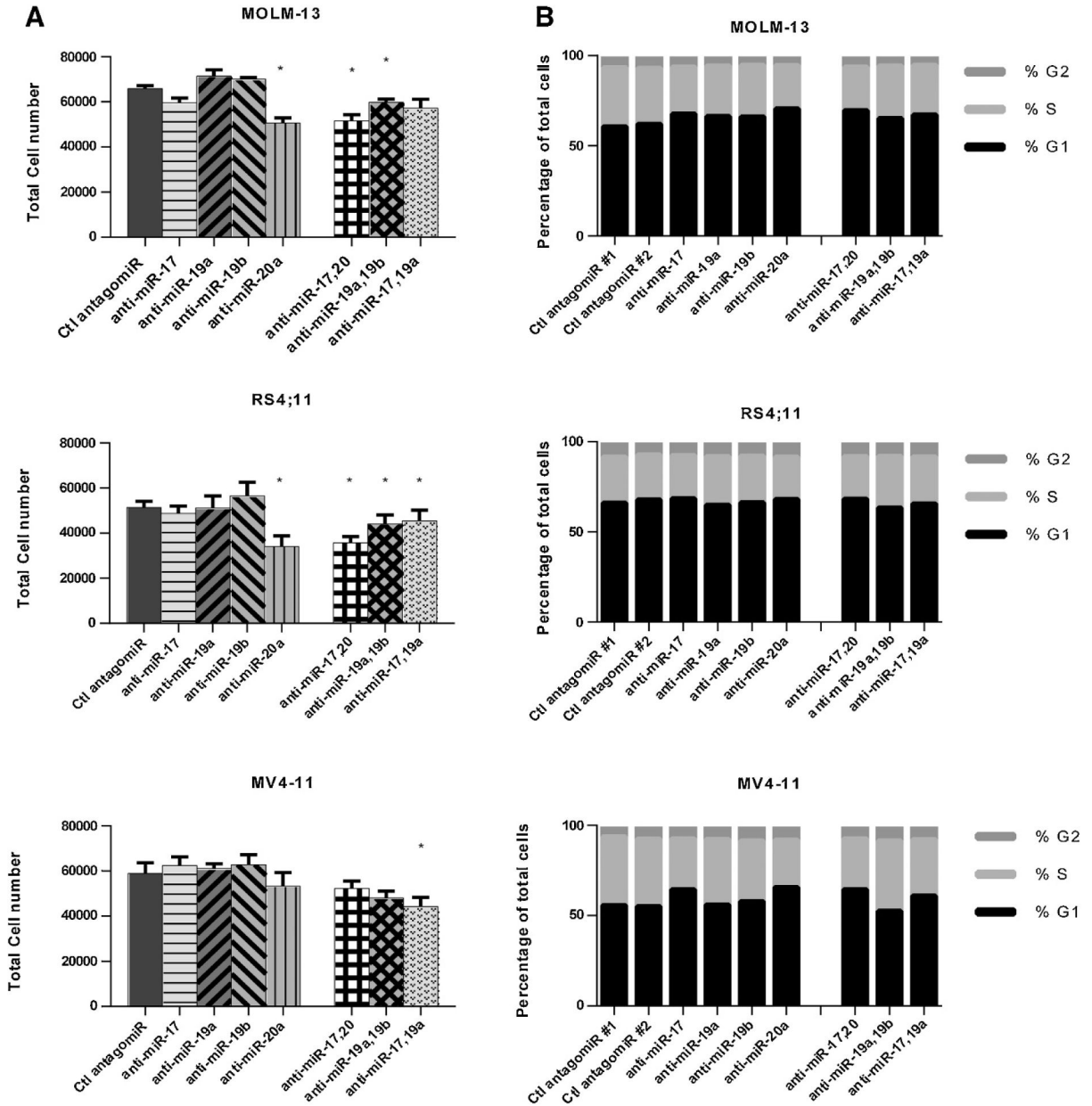


Figure 3. Antagomir treatment of *MLL* leukemia cell lines in liquid culture causes a modest decrease in total cell number and variable accumulation at G1

A) Total cell number was assessed using Cell-Titer-Glo assay after 4 days. Antagomir treatment was performed in three independent experiments in triplicate (1 uM total concentration). Statistical significance was tested against a control antagomir treatment (* = $p < 0.05$) using Student's t-test. *Anti-Ce-miR-67* was used as a control for RS4;11, and *anti-miR-10a* was used as a control for MOLM-13 and MV4-11. B) Cell cycle distribution was determined by propidium iodide (PI) staining from three independent experiments for each cell line.

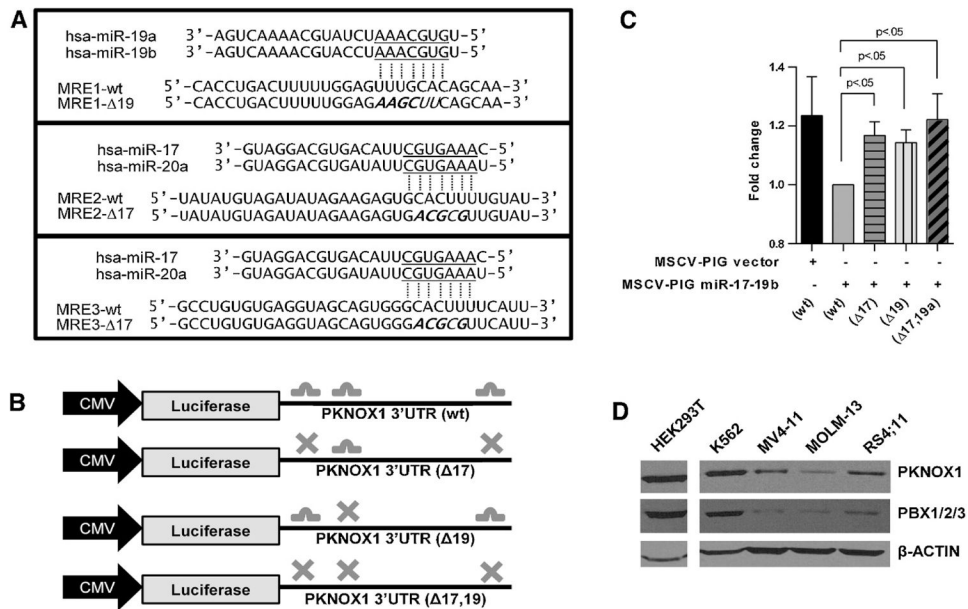


Figure 4. Pknox1 is a valid target of the miR-17~92 cluster

A) Putative miRNA response element mutations in *PKNOX1* 3'UTR. Nucleotide changes for mutant constructs are indicated with underlines. B) Schematic of luciferase reporter construct design and mutation placement. C) Luciferase response relative to wild type control. Luciferase levels were examined in 4 independent experiments. Statistical significance was tested against wt ($p < .05$) using student's t-test. D) Endogenous levels of PKNOX1 and PBX1/2/3 were examined in human cell lines. Unnecessary lanes cropped out.

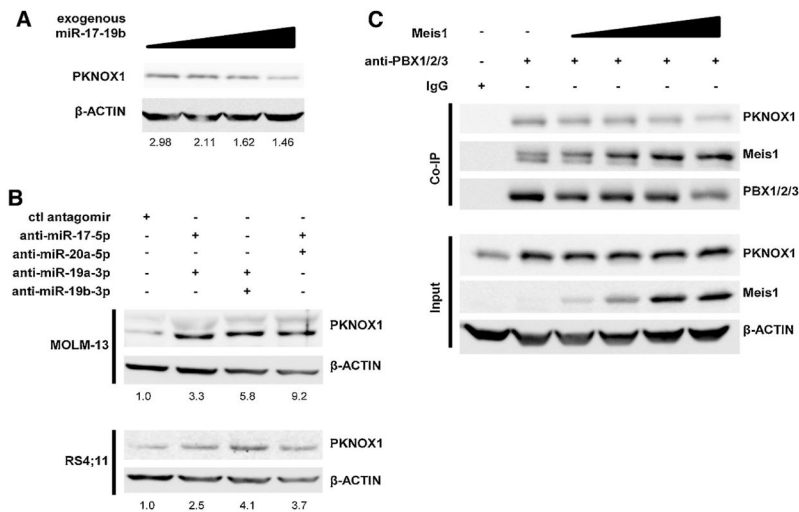


Figure 5. Antagomir treatment results in increased PKNOX1 levels and participation in HOX-PBX complexes

A) PKNOX1 protein levels in HEK293T cells were measured in response to transfection with increasing concentrations of a plasmid expressing *miR-17~19b* with quantification of PKNOX1 levels relative to loading control indicated below blot. B) Human leukemia cell lines were treated with antagomirs (2 μ M for 72 hrs) and analyzed for PKNOX1 protein levels by western blot. B) HEK293T cells were transfected with *Meis1* expressing plasmid and subjected to co-immunoprecipitation for PBX1/2/3. 20% input (bottom) was subject to western blotting for PKNOX1 and MEIS1. Co-immunoprecipitation was performed using antibody against PBX1/2/3. Representative blot depicted from experiment were performed in triplicate.

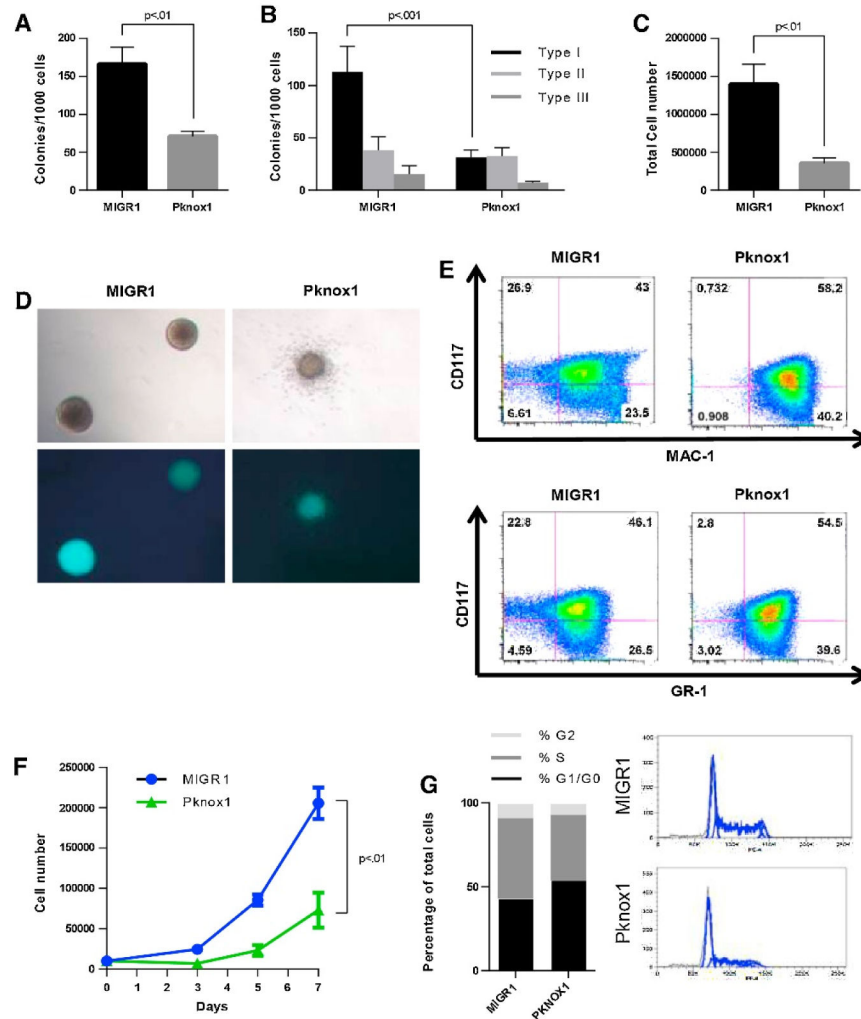


Figure 6. Enforced Pknox1 expression inhibits *MLL-AF9* colony forming ability and proliferation

MLL-AF9 transformed murine bone marrow cells were infected with either *MIGR1* or a *Pknox1* expressing virus. Cells were plated in methylcellulose to assess colony forming ability (A – E) or in liquid culture to assess proliferation (F–G) for 1 week in 4 independent experiments. A) Colony number was assessed and B) colony type were examined in all experiments. Statistical analysis was performed using student's t-test. C) Total cell numbers in colony assays were determined from cells collected from colony assays. D) Colony morphology (top) and GFP expression (bottom) were examined. E) Surface marker staining was assessed for CD117, MAC-1, and GR-1 in 4 independent experiments. F) Total cell number was calculated against a standard curve using Cell Titer Glo Assay from cells in liquid culture. Statistical significance was determined using student's t-test. G) Cell cycle distribution was analyzed by FACS using propidium iodide staining. Distribution of live cells was averaged from 3 independent experiments (left). Example FACS plots are shown (right).