RESEARCH ARTICLE

CELL BIOCHEMISTRY & FUNCTION WILEY

MiR-27a downregulates 14-3-3θ, RUNX1, AF4, and MLL-AF4, crucial drivers of blast transformation in t(4;11) leukemia cells

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Funding information
Italian Ministry of Health [RF-2011-02349269 to GE]; Italian Ministry of University and Research ["Fondo di finanziamento per le attività base di ricerca" (FFABR) 2017] to AC

Abstract

The chromosomal translocation t(4;11)(q21;q23), a hallmark of an aggressive form of acute lymphoblastic leukemia (ALL), encodes mixed-lineage leukemia (MLL)-AF4 oncogenic chimera that triggers aberrant transcription of genes involved in lymphocyte differentiation, including *HOXA9* and *MEIS1*. The scaffold protein 14-3-3θ, which promotes the binding of MLL-AF4 to the *HOXA9* promoter, is a target of MiR-27a, a tumor suppressor in different human leukemia cell types. We herein study the role of MiR-27a in the pathogenesis of t(4;11) ALL. Reverse transcription quantitative PCR (qPCR) reveals that MiR-27a and 14-3-3θ expression is inversely correlated in t(4;11) ALL cell lines; interestingly, MiR-27a relative expression is significantly lower in patients affected by t(4;11) ALL than in patients affected by the less severe t(12;21) leukemia. In t(4;11) leukemia cells, ectopic expression of MiR-27a decreases protein level of 14-3-3θ and of the key transcription factor RUNX1. We show for the first time that MiR-27a also targets AF4 and MLL-AF4; in agreement, MiR-27a overexpression strongly reduces AF4 and MLL-AF4 protein levels in RS4;11 cells. Consequent to AF4 and MLL-AF4 downregulation, MiR-27a overexpression negatively affects transcription of *HOXA9* and *MEIS1* in different t(4;11) leukemia cell lines. In agreement, we show through chromatin immunoprecipitation experiments that MiR-27a overexpression impairs the binding of MLL-AF4 to the *HOXA9* promoter. Lastly, we found that MiR-27a overexpression decreases viability, proliferation, and clonogenicity of t(4;11) cells, whereas it enhances their apoptotic rate. Overall, our study identifies the first microRNAthat strikes in one hit four crucial drivers of blast transformation in t(4;11) leukemia. Therefore, MiR-27a emerges as a new promising therapeutic target for this aggressive and poorly curable form of leukemia.

KEYWORDS

14-3-3θ, AF4, microRNA, MiR-27a, MLL, t(4;11) acute leukemia, target therapy

Tiziana Fioretti and Mariateresa Zanobio contributed equally to this study.

1 | INTRODUCTION

Genomic rearrangements involving the *mixed lineage leukemia* (*MLL* aka *KMT2A*) gene cause 5%–10% of childhood acute lymphoblastic leukemia (ALL) cases.[1] The translocation t(4;11)(q21;q23) connects the *MLL* with the *AFF1* gene and produces a fusion transcript that encodes the MLL-AF4 oncogenic chimera. It has been found in about 50% of affected infants with *MLL* rearrangements and gives rise to an aggressive leukemia form, characterized by early relapse and dismal prognosis.[1–3]

The *AFF1* gene encodes AF4, a member of the AF4 protein family. The AF4 family includes AF5q31 and LAF4, which also are among the *MLL* fusion partners in leukemia cases.[2,3] Notably, the AF4 family members interact with the ENL family members and with the P-TEFb elongation factor and form the AEP complex.[3] The MLL-AF4 oncoprotein constitutively recruits the AEP complex and aberrantly activates transcription of key genes involved in lymphocyte differentiation, including the homeobox A (*HOXA*) cluster genes and *MEIS1*.[4–8] Recruitment of AEP complex on target genes triggers the aberrant activity of the histone H3 lysine-79 (H3K79) methyltransferase DOT1L, which tags actively transcribed genes with the epigenetic signature H3K79me2, H3K27ac, and H3K4me3, which are a hallmark of MLL-AF4 target genes.[7,9] Ultimately, the oncogenic potential of MLL-AF4 is mostly driven by the interaction with AF4 and its protein partners.

Among the numerous proteins belonging to the AF4 interactome, there is the scaffold protein 14-3-3θ, which affects MLL-AF4 aberrant activity through a recently elucidated regulatory mechanism.[10,11] In particular, through the direct interaction with AF4, 14-3-3θ acts as an oncogene with an important role in the MLL-AF4 leukemic transformation. Notably, 14-3-3θ knockdown decreases MLL-AF4 target gene expression, induces apoptosis, and hampers proliferation of t(4;11) leukemia cells.[11]

A negative regulator of 14-3-3θ is MiR-27a.[12] MicroRNAs (MiRs) are evolutionary conserved, small noncoding RNA molecules that posttranscriptionally regulate gene expression by specific binding to 3′-untranslated region (UTR) of their targets. Increasing evidence indicates that MiR-27a plays a key role in tumor biology, including tumorigenesis, proliferation, apoptosis, invasion, migration, and angiogenesis. Moreover, MiR-27a has clinical significance in drug sensitivity, treatment of cancer, and patient prognosis.[12–15] Various studies reveal that MiR-27a is significantly dysregulated in different cancers. In non-small-cell lung cancer, liver cancer, colon cancer, and prostate cancer, it acts as an oncogene, whereas it is a tumor suppressor in gastric cancer, bladder cancer, and esophageal squamous cell, as well as in leukemia.[12–15] In particular, MiR-27a expression is downregulated in several pre-B- and T-ALL cell lines, where it functions as a tumor suppressor also through the negative regulation of 14-3-3θ. Moreover, MiR-27a levels are low in primary patient samples, and its cellular replacement results in decreased cell growth and increased cell death.[12]

On this basis, we herein analyze the role of MiR-27a in the molecular pathogenesis of the rare and poorly curable t(4;11) leukemia.

Significance statement

A growing body of evidence indicates that microRNAs targeting genes involved in modulating hematopoietic process are dysregulated t(4;11) acute leukemia, a very aggressive and refractory hematologic cancer. Here, we show that MiR-27a is downregulated both in t(4;11) leukemia cell lines and primary patient samples. We demonstrate that MiR-27a targets four crucial drivers of blast transformation, namely, 14-3-3θ, RUNX1, AF4, and mixed-lineage leukemia (MLL)-AF4 in t(4;11) leukemia cells. Thus, MiR-27a, by striking these crucial oncogenes, functions as a tumor suppressor in t(4;11) leukemia and emerges as a new potential target for the therapy of this still poorly treatable form of leukemia.

2 | MATERIALS AND METHODS

2.1 | Antibodies

Anti-14-3-3θ, sc-632; anti-α-tubulin, sc-5286; anti-vinculin, sc-25336 (Santa Cruz Biotechnology); anti-AF4, #A302-344A (Bethyl Laboratories); anti-MLL^N, #05-764; anti-MLL^C, #05-765 (Merck Millipore); anti-RUNX1, #4336 (Cell Signaling Technology); horseradish peroxidase-conjugated anti-mouse (#NA931) and anti-rabbit (#NA934) IgG secondary antibodies (GE Healthcare).

2.2 | Cell lines

Leukemia cell lines were obtained from the Cell Culture Facility of CEINGE—Advanced Biotechnologies and from IRCCS SDN (Naples, Italy). RS4;11 ALL cells were grown in minimum essential medium (MEM) (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS) (Lonza); SEM ALL cells in Iscove's modified Dulbecco's medium (Lonza) supplemented with 10% FBS; MV4-11 acute monocytic leukemia (AML), 697 ALL, REH B-cell precursor leukemia, and HL-60 AML cells in RPMI supplemented with 20% FBS and 10 ml/L penicillin/streptomycin (Sigma-Aldrich). RS4;11, SEM, and MV4-11 harbor the t(4;11) chromosomal rearrangement. REH harbors the t(12;21) chromosomal rearrangement.

2.3 | Molecular cloning and site-directed mutagenesis

The *AFF1*-3′-UTR was amplified from genomic DNA in three overlapping fragments, by using specific primer pairs (Supporting Information: Figure S1 and Table S1). Primers used to amplify the central fragment of *AFF1*-3′-UTR (584 bp) are as follows: F—*Xho*I: 5′-GCCGC/TCGAGTTCCCAAAGGCAAAATCTGT-3′ and R—*Not*I:

5′- GCCGGC/GGCCGCATAAGTGCGGTCCAATCTGT-3′. Amplified fragments were cloned in psiCHECK2™ vector (#C8021; Promega) after digestion with *SgF*1 #R7103 (Promega) or *Xho*I #10703770001, and *Not*I #11014714001 (Roche). Mutant psiCHECK2-*AFF1*-3′-UTR construct was obtained by QuickChange Mutagenesis Kit (#200518; Agilent) and primers: forward, 5′-GTGTTTAATGTTTCTGTCCTTTA TCTGTATTATTGAATTTAAGAGCCCTGC-3′ and reverse, 5′-GCA GGGGCTCTTAAATTCAATAATACAGATAAAGGACAGAAACATTAAA CAC-3′.

2.4 | Cell transfection

Hsa-pre-miR-27a-3p- (#AM17100, assay ID PM10939; Ambion) or has-pre-miR-negative control (#AM17110; Ambion), and hsa-anti-miR-27a-3p- (#AM17000, assay ID AM10939; Ambion) or has-anti-miR-negative control (#AM17010; Ambion) was added to 1×10^7 cells at a final concentration of 50 nM in 0.1 ml of opti-MEM (Thermo Fisher Scientific Inc.). Cells were transfected by electroporation.

2.5 | RNA isolation, reverse transcription, and quantitative PCR

Total RNA from the bone marrow of nine patients affected by t(4;11) ALL and seven patients with t(12;21) ALL was obtained from the CEINGE Biobank. All patients signed informed consent and agreed to use their samples for scientific research purposes, in an anonymous form. The Ethics Committee of the University of Naples Federico II approved the study (protocol code 77/21, March 26, 2021).

Total RNA was extracted with an RNeasy Mini Kit (ID: 74104; Qiagen). MiR-27a was reverse-transcribed (RT) with a small RNA–specific primer and TaqMan MicroRNA Reverse Transcription Kit (#4366596, Thermo Fisher Scientific Inc.). Complementary DNA (cDNA) was synthesized with SuperScript™III First-Strand Synthesis System (#12574; Thermo Fisher Scientific Inc.).[16]

MiR-27a quantitative PCR (qPCR) was performed in TaqMan® Universal Master Mix II with 1 μl of TaqMan probe (TaqMan® MicroRNA Assays; Thermo Fisher Scientific Inc.), specific for MiR-27a (#4427975, assay ID 000408) or for U18 small nucleolar RNA (#4351372, assay ID Hs06637271_g1). MiR-27a relative expression was normalized to U18. qPCR of gene-specific cDNA was performed with iQ™ SYBR® Green Supermix (#1708880, Bio-Rad Laboratories s.r.l.) and gene-specific primer pairs.[11,17] Relative gene expression was normalized to *POLR2A* and *ACTB*. Data were analyzed with $2^{-\Delta\Delta C_t}$ methods.

For patients' data analysis, relative quantification of normalized gene expression was determined using the Bio-Rad CFX Manager Gene Expression tool (Bio-Rad Laboratories); machinery tool information: normalized expression mode → $2^{-\Delta\Delta C_q}$, without the control sample.[18,19] Relative gene expression was normalized to *POLR2A* and *ACTB*. Graphically reported values were averages from at least three independent experiments.

2.6 | Western blot analysis

Whole protein extracts (WCEs) were prepared from leukemia cells incubated in the lysis buffer (50 mM Tris/HCl pH 8, 150 mM NaCl, 0.5% NP-40, 2 mM EDTA pH 8, 1 mM Na_3VO_4, 10 mM NaF), and 0.1% (v/v) protease inhibitor cocktail (Sigma-Aldrich). WCEs were clarified at 16,000*g* and protein concentration was measured by Bio-Rad protein assay (#5000001; Bio-Rad Laboratories). Forty micrograms of WCEs were loaded, resolved on 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto the nitrocellulose membrane. Membranes were incubated with appropriate primary and secondary antibodies, and protein signals were detected with the ECL Plus detection system (#RPN2132; GE Healthcare) and quantified by a scanner equipped with a sun spark classic densitometric workstation.[20,21]

2.7 | Bioinformatic analysis

MiRNA putative seed sequence was predicted by using TargetScan,[22] microRNA.org (www.microrna.org), DIANA Tools' softwares (http://carolina.imis.athena-innovation.gr).

2.8 | Luciferase reporter assay

RS4;11 cells ($1 \times 10^{7)}$ were cotransfected with pre-MiR-27a and the psiCHECK2-*AFF1*-3′-UTR constructs (each with the appropriate controls). After 24 h, cells were harvested and assays were performed using the Dual-Luciferase® Reporter (DLR) Assay System (#16185; Thermo Fisher Scientific Inc.).

2.9 | Chromatin immunoprecipitation (ChIP)

RS4;11 cells (30×10^6) were transfected with pre-MiR-27a or pre-MiR-negative control and, after 36 h, fixed in optiMEM containing 1% formaldehyde; the reaction was stopped by glycine quenching (125 mM). ChIP and DNA extraction, as well as qPCR, were performed as already described.[17,23] A pool of nonspecific IgG was used as ChIP-negative control; *ACTB* gene promoter (*ACTB* pr) represented the negative control chromatin.

2.10 | Cell viability assay

The proliferation of cells transfected with pre-MiR-27a or pre-MiR-negative control was assessed using the Cell Counting Kit 8 (CCK8) (#CK04-11; Dojindo EU GmbH). Absorbance at 540 nm was measured 24, 48, and 72 h after transfection, for each analyzed cell line, using a Spectramax spectrophotometer (Molecular Devices). Cell viability was normalized versus nonelectroporated (untransfected) cells. The percentage of viable cells is expressed as:

Viability: [(sample well mean OD − background mean OD)
/(control well mean OD − backgroundmean OD)] × 100%.

2.11 | 5-Bromo-2′-deoxyuridine (BrdU) cell proliferation assay

Forty-eight hours after transfection with pre-MiR-27a or pre-MiR-negative control, cells ($1 × 10^6$/ml) were treated with 1 mM BrdU, fixed, permeabilized, and stained with a fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody and with 7-aminoactinomycin D (#559619; BD Pharmingen). Cytofluorimetric analysis was carried out by FACS.

2.12 | Apoptosis assay

Seventy-two hours after transfection with MiR-27a or pre-MiR-negative control, cells ($4 × 10^6$) were incubated in annexin binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$) and centrifuged at 300g at 4°C for 10 min. A solution of Annexin V-FITC (#130-092-052; Miltenyi Biotec) and propidium iodide (#556463, BD Pharmingen) was added. Positive staining was detected by FACS.

2.13 | Colony formation assay

Twenty-four hours after transfection with MiR-27a or pre-MiR-negative control, cells ($1.2 × 10^4$) were seeded in MethoCult™ (#H4034; STEMCELL Technologies). After 2 weeks, colony morphology and number were determined under an inverted microscope (Leica, Germany), with a ×20 objective.

2.14 | Statistical analysis

All data are expressed as mean ± SD and are representative of three or more independent experiments. The data of repeated experiments were analyzed using one-way two-tailed paired Student's t-test. p Values were presented as *<.05, **<.01, and ***<.001. Pearson's R value was calculated, for all the tested cell lines, to assess the correlation between MiR-27a and 14-3-3θ transcript levels (Supplementary Figure S2)

3 | RESULTS

3.1 | MiR-27a regulates the expression of 14-3-3θ and RUNX1 in t(4;11) leukemia cells

We performed RT-qPCR analysis to compare transcript levels of MiR-27a and 14-3-3θ in different ALL and AML cell lines, and in peripheral blood lymphocytes from healthy donors. We observed relatively low expression of MiR-27a in the ALL cells (Figure 1A), and relatively high transcript levels of 14-3-3θ only in t(4;11) cells. In agreement, the value of Pearson's $R = −.961 ± .032$ indicated a strong inverse correlation between MiR-27a and 14-3-3θ expression in t(4;11) cell lines (Supporting Information: Figure S2); notably, MiR-27a expression was relatively higher in MV4-11, the t(4;11) AML cell line, with respect to RS4;11 and SEM, which are t(4;11) ALL cell lines (Figure 1A).

Low MiR-27a expression levels correlate with poor prognosis and low relapse-free survival in ALL patients.[24] Therefore, we evaluated MiR-27a and 14-3-3θ transcript levels, by RT-qPCR, in lymphocytes of patients with t(4;11) and of patients with t(12;21) (p13;q22) translocation, an indicator of good prognosis.[25] Notably, MiR-27a levels were significantly lower in t(4;11) than in t(12;21) patients (Figure 1B, left). In contrast, although 14-3-3θ transcript levels were slightly higher in t(4;11) than in t(12;21) patients, the difference was not statistically significant (Figure 1B, right). Also, Pearson's R value did not confirm the inverse correlation between MiR-27a and 14-3-3θ expression previously observed in t(4;11) leukemia cell lines, likely because other mechanisms can influence 14-3-3θ expression in the patients. However, despite interindividual variability and the relatively small number of patients, these overall results supported the relationship between low MiR-27a expression and poor prognosis in t(4;11) ALL.

To test whether MiR-27a actually targeted 14-3-3θ also in t(4:11) leukemia, we transiently transfected RS4;11 ALL cells with pre-MiR-27a or the pre-MiR-negative control and evaluated 14-3-3θ protein expression. Western blot analysis indicated that MiR-27a overexpression (Supporting Information: Figure S3A) decreased the 14-3-3θ level (Figure 1C), thereby confirming the inverse relationship existing between 14-3-3θ and MiR-27a in basal conditions. An additional known target of MiR-27a with a key role in t(4;11) leukemia is the transcription factor RUNX1.[4,26–28] In agreement with and similar to 14-3-3θ, MiR-27a overexpression significantly decreased the RUNX1 level (Figure 1D).

3.2 | AF4 and MLL-AF4 are novel targets of MiR-27a

To look for new potential MiR-27a targets involved in t(4;11) leukemia, we interrogated various miRNA prediction tools that detected a 7-mer seed sequence in 3′-UTR of the *AFF1* gene, 2884–2890 nucleotides downstream of the stop codon. Since *AFF1* and *KMT2A/AFF1* share their 3′-UTR, we considered also the fusion transcript a putative target of MiR-27a.

We therefore evaluated AF4 and MLL-AF4 protein levels in RS4;11 cells that expressed ectopic MiR-27a and found a strong decrease in both AF4 and MLL-AF4 protein amount (Figure 2A). In agreement, RS4;11 cells transfected with anti-MiR-27a, which binds MiR-27a and inhibits its function, showed a twofold increase of AF4
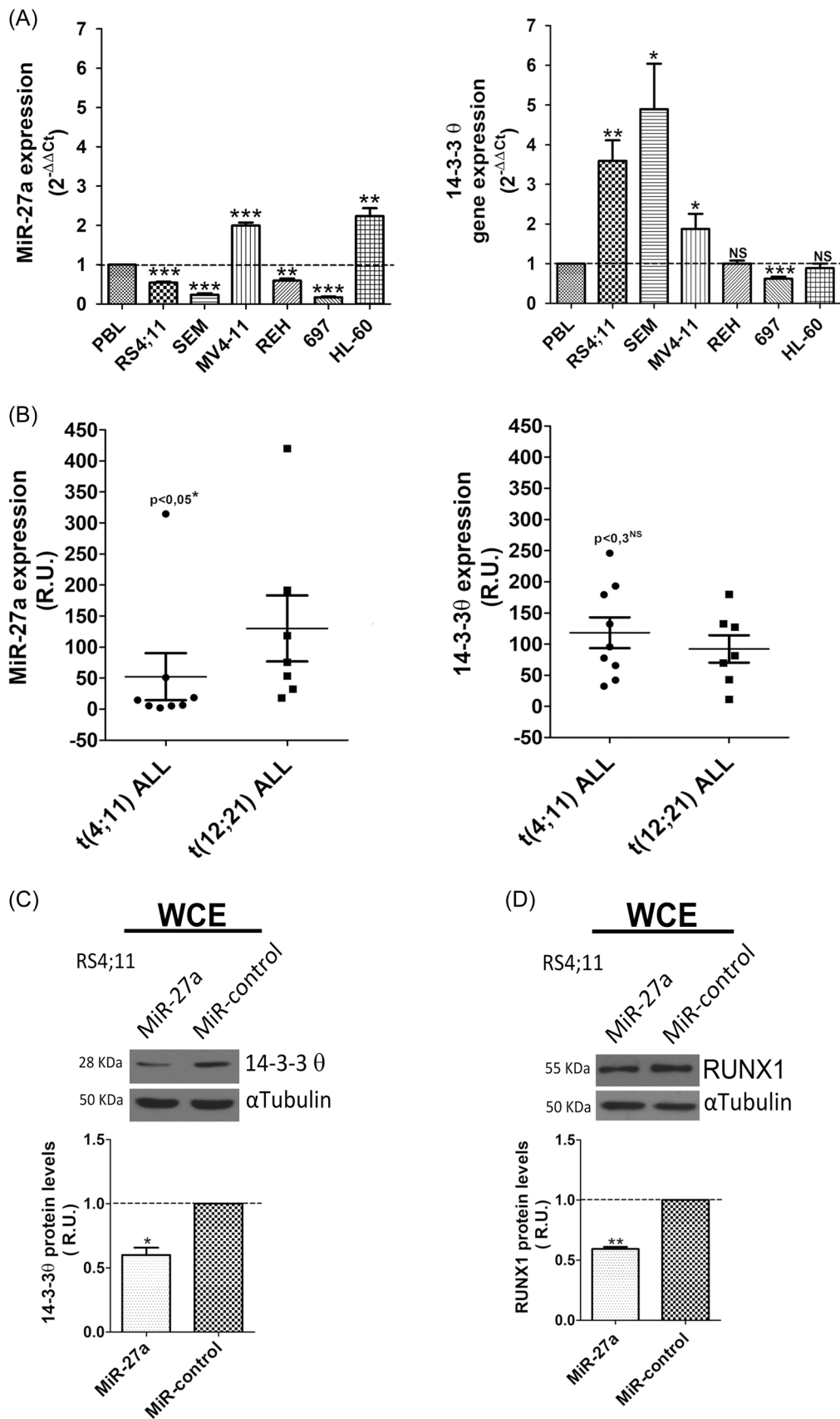
FIGURE 1 (See caption on next page)

and MLL-AF4 protein amounts with respect to the control cells (Figure 2A).

Notably, MiR-27a overexpression decreased the *AFF1* transcript level by about 40%, thereby suggesting posttranscriptional repression of *AFF1* through messenger RNA destabilization rather than translation inhibition (Figure 2B). Since *AFF1* and *KMT2A/AFF1* share their 3′-UTR, we assumed MiR-27a repressed MLL-AF4 through the same mechanism.

To functionally identify MiR-27a seeds within the *AFF1* transcript, as previously reported for RUNX1 and 14-3-3θ, we performed DLR assays.[12,26] Three different constructs, covering the whole *AFF1*-3′-UTR (Supporting Information: Figure S1 and Table S1), were cotransfected with pre-miR-27a or pre-MiR-negative control in RS4;11 cells. Construct 2-*AFF1*-3′-UTR, which contained the predicted 7-mer seed identified by bioinformatic analysis, caused a significant decrement in *Renilla* activity (Supporting Information: Figure S4). Therefore, we subcloned a 584-bp-long fragment (*AFF1*-3′-UTR wild type [WT]) that contained this 7-mer seed and generated the corresponding negative control, *AFF1*-3′-UTR mutant (MUT), by site-directed mutagenesis (Figure 2C). *AFF1*-3′-UTR WT transfection decreased *Renilla* activity by about 50%, whereas no effect was observed with *AFF1*-3′-UTR MUT (Figure 2C).

3.3 | MiR-27a affects MLL-AF4 chimera's activity in t(4;11) cell lines

14-3-3θ, RUNX1, AF4, and MLL-AF4 have a crucial role in t(4;11)-driven leukemic transformation through deregulation of gene expression.[11,29] We found that MiR-27a transfection (Supporting Information: Figure S3) significantly decreased *HOXA9*, *HOXA7*, and *MEIS1* transcript levels in different t(4;11) cell lines (Figure 3A). This effect was specific because MiR-27a overexpression did not reduce the transcript level of these genes in REH cells, a non-MLL-related ALL cell line (Figure 3A).

We performed ChIP to evaluate the strength of MLL-AF4 binding to the *HOXA9* promoter (*HOXA9* pr), after transfection of pre-miR-27a or pre-MiR-negative control, in RS4;11 cells. We used an anti-MLL[N] antibody, targeting both WT MLL and MLL-AF4 chimera, and an anti-MLL[C] that targeted WT MLL. By qPCR, we measured the amount of *HOXA9* pr and *ACTB* promoter (chromatin negative control) in DNA immunoprecipitated with anti-MLL[N] and anti-MLL[C].[11,17,23] Percentage of *HOXA9* pr in chromatin immunoprecipitated with anti-MLL[N] was significantly lower in cells transfected with pre-MiR-27a compared to

the negative control cells (Figure 3B); the absence of *ACTB* demonstrated the assay specificity.

3.4 | MiR-27a affects cell viability, proliferation, apoptosis, and clonogenic capacity of t(4;11) leukemic cells

We showed that MiR-27a targeted a set of relevant genes for MLL-AF4-driven leukemia cell transformation. Consistently, we also showed, using MTT assay, that MiR-27a overexpression decreased the viability of the tested t(4;11) cells by at least 20% (Figure 4A). Furthermore, in the representative RS4;11 ALL cell line, 48 h after pre-MiR-27a transfection, BrdU assay showed, coherently with cell viability results, decreased number of cells in the S phase of the cell cycle, that is, proliferating cells (Figure 4B).

HOXA9 knockdown induces apoptosis in the MLL-leukemia cell lines,[30] and 14-3-3 proteins belong to a family of antiapoptotic proteins.[31] To test whether overexpression of MiR-27a had a proapoptotic effect, we performed apoptosis assays in RS4;11 cells transfected with MiR-27a. Seventy-two hours after transfection, the apoptotic rate of cells transfected with MiR-27a was about twofold higher than in MiR-control cells (Figure 4C). We also tested whether MiR-27a overexpression affected the clonogenic capacity of t(4;11) cells through colony formation assay. After 2 weeks in culture, colonies of cells transfected with pre-MiR-27a were smaller and less numerous than the control ones (Figure 4D).

4 | DISCUSSION

In t(4;11) leukemia, the oncogenic chimera MLL-AF4 is necessary and sufficient to activate hematopoietic progenitor transformation.[28,32] In many patients, survival of leukemic blasts depends on the maintenance of high expression levels of a set of MLL-AF4 target genes, including *HOXA9*, *HOXA8*, *HOXA7*, and *MEIS1*[6]; notably, *HOXA9* silencing alone is sufficient to promote apoptotic death of t(4;11)(q21;q23) lymphoblasts.[33]

To exert its aberrant function, MLL-AF4 exploits numerous protein interactors, with very heterogeneous cellular roles, that acquire an opportunistic oncogenic function.[11,23] As a consequence, a wide plethora of proteins are dysregulated in the leukemia context.[34,35]

MiRNAs are a class of small noncoding RNAs that regulates gene expression in eukaryotes. More importantly, a single miRNA

FIGURE 1 MiR-27a affects the expression of 14-3-3θ and RUNX1 in t(4;11) leukemia cells. (A) MiR-27a and 14-3-3θ transcript levels in different leukemia cell lines; PBLs are used as reference. Relative gene expression was determined by the $2^{-\Delta\Delta C_t}$ method. (B) MiR-27a and 14-3-3θ transcript level in t(4;11) versus t(12;21) ALL patients. Relative gene expression was determined by the $2^{-\Delta\Delta C_q}$ method, without the control sample. Western blot analysis of (C) 14-3-3θ and (D) RUNX1 protein level in RS4;11 cells transfected with MiR-27a or MiR-negative control; αTubulin, loading control. Error bars indicate standard deviations of triplicate experiments. MiR, microRNA; NS, not statistically significant; PBL, peripheral blood leukocytes; R.U., relative units; WCE, whole-cell extract. *$p < .05$; **$p < .01$; ***$p < .005$.
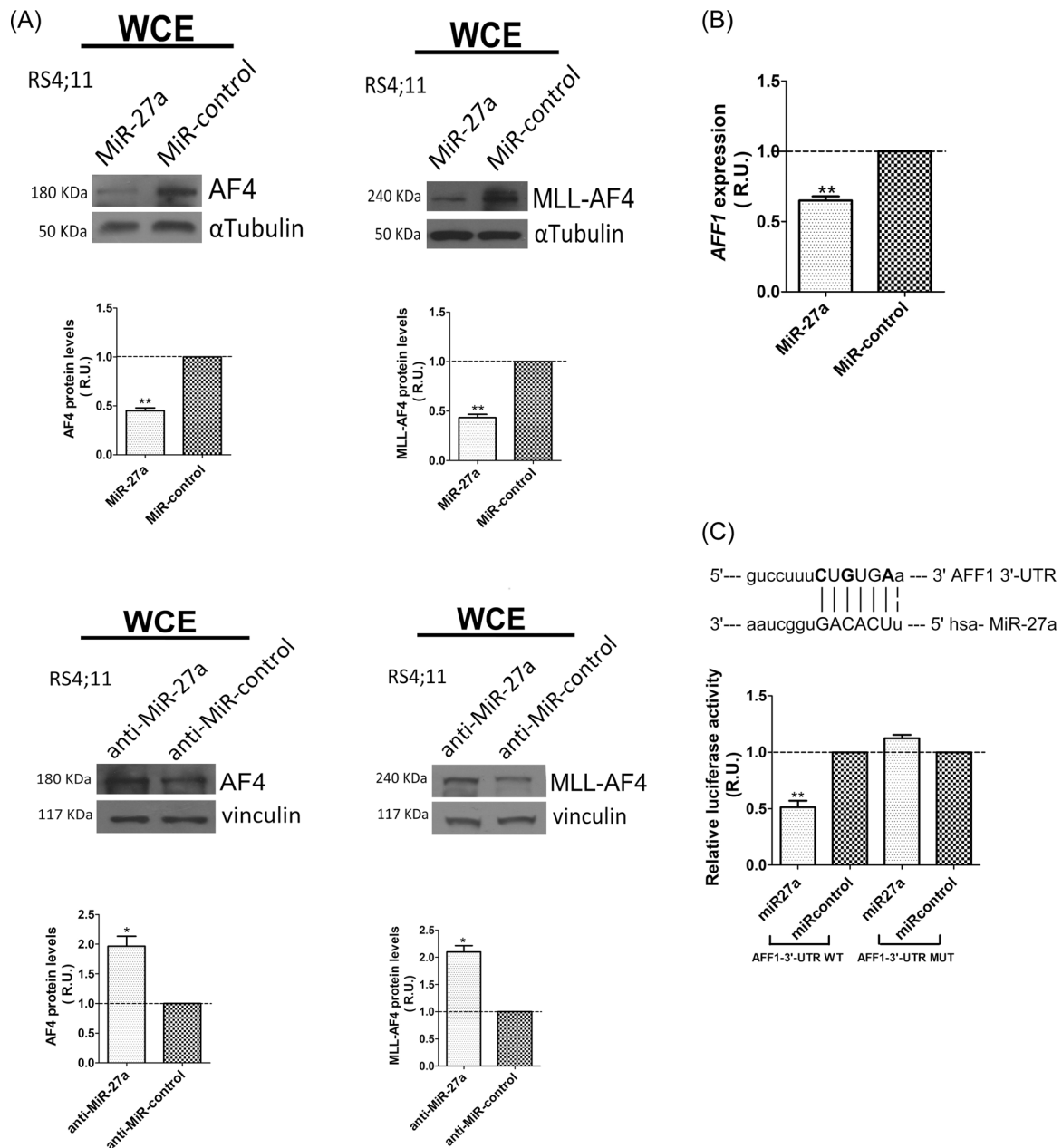
FIGURE 2 AF4 and MLL-AF4 are novel targets of MiR-27a. (A) Western blot analysis of AF4 and MLL-AF4 levels in RS4;11 cells transfected with MiR-27a and with MiR-negative control; αTubulin, loading control (upper panel). Western blot analysis of AF4 and MLL-AF4 levels in RS4;11 cells transfected with anti-MiR-27a and the anti-MiR-negative control; vinculin, loading control (lower panel). (B) *AFF1* transcript levels were measured 24 h after cell transfection with MiR-27a or MiR negative control. Relative gene expression was determined by the $2^{-\Delta\Delta C_t}$ method. (C) Sequence alignment of MiR-27a and its putative seed within *AFF1*-3′-UTR, and dual-luciferase reporter assay results in RS4;11 cells cotransfected with MiR-27a and the psiCHECK-2 vector containing *AFF1*-3′-UTR WT or MUT, and the relative controls. In bold are the nucleotides mutated in the MUT construct. Error bars indicate standard deviations. miR, microRNA; MLL, mixed-lineage leukemia; MUT, mutant; R.U., relative units; UTR, untranslated region; WCE, whole cell extract; WT, wild type. *$p < .05$; **$p < .01$.

can regulate very large sets of genes, thereby influencing almost every metabolic and regulatory pathway in individuals who are healthy as well as disease.[15,36–38] A growing number of MiRNAs that target genes involved in the hematopoietic process are dysregulated in human leukemia.[39–42] In t(4;11) ALL, MLL-AF4 activates aberrant expression of the oncogenic miR-130b and miR-128a;[43] in contrast, the tumor suppressor miR-142-3p,

miR-142-3p, and miR-205 target and downregulate MLL-AF4 and AF4.[44–46]

The scaffold protein 14-3-3θ, a direct interactor of AF4 and MLL-AF4, acquires an opportunistic oncogenic role in t(4;11) leukemia. Indeed, 14-3-3θ knockdown negatively affects *HOXA9* and *MEIS1* expression, induces apoptosis, and hampers proliferation of cells that constitutively express MLL-AF4.[11] Intriguingly, MiR-27a
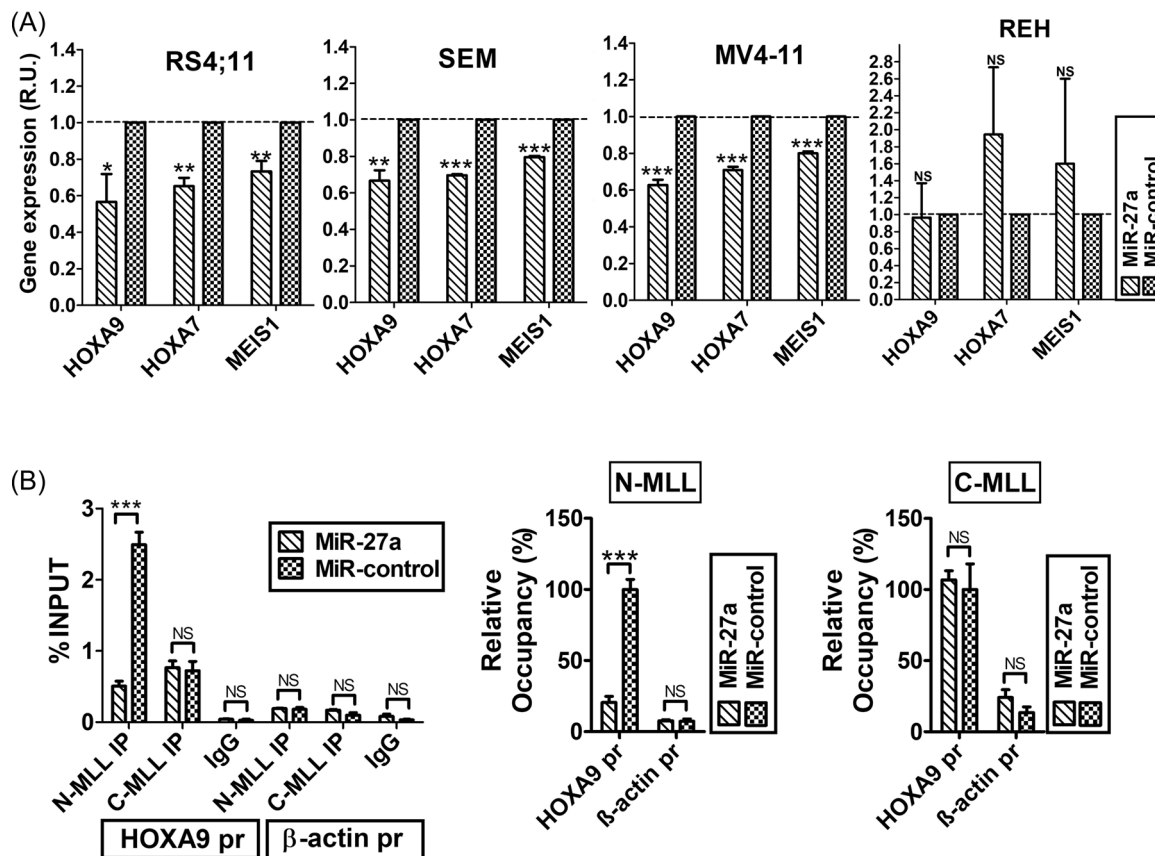
FIGURE 3 MiR-27a impairs the expression of MLL-AF4 target genes in t(4;11) cell lines. (A) *HOXA9*, *HOXA7*, and *MEIS1* transcript expression in RS4;11, SEM, MV4-11, and REH, 24 h after transfection with MiR-27a or MiR-negative control. Relative gene expression was determined by the $2^{-\Delta\Delta C_t}$ method. (B) Interaction of MLL-AF4 and wild-type MLL with *HOXA9* gene promoter (HOXA9 pr) after RS4;11 cell transfection with MiR-27a or MiR negative control, determined by ChIP. *ACTB* promoter (β-actin pr) is the negative control. Data are expressed as a percentage of *HOXA9* and *ACTB* promoters in immunoprecipitated chromatin with respect to the INPUT. IgG is the mock (left panel). Error bars indicate standard deviations. ChIP, chromatin immunoprecipitation; MLL, mixed-lineage leukemia; NS, not statistically significant; R.U., relative units. *p< .05; **p< .01, ***p < .005.

functions as a tumor suppressor in different acute leukemia forms by downregulating 14-3-3θ; moreover, it is downregulated in leukemia cell lines and in patients' primary samples compared to hematopoietic progenitor cells.[12]

Interestingly, we found a statistically significant inverse correlation between transcript levels of MiR-27a and 14-3-3θ in SEM and RS4;11, the t(4;11) ALL cell lines, where relatively low levels of MiR-27a associated with relatively high expression of 14-3-3θ (Figure 1A). Notably, despite Pearson's value revealing an inverse correlation between MiR-27a and 14-3-3θ expression also in MV4-11, MiR-27a level was higher in t(4;11) AML cell line compared to SEM and RS4;11. This observation is in agreement with previous expression microarray analysis performed on a large number of leukemia samples, which found no significant differences in MiR-27a expression between CD34+ hematopoietic stem/progenitor cells and AML cases.[12]

MiR-27a is expressed at lower levels tends to relapse in infant and childhood ALL patients compared with primary patients at diagnosis; in contrast, patients with high levels of MiR-27a

at diagnosis have longer relapse-free survival than those with low levels, suggesting that MiR-27a dysregulation might contribute to leukemia relapse.[24,47] In ALL, the t(12;21) translocation is a good-risk prognostic biomarker, whereas t(4;11) has a very poor prognosis.[48–50] Interestingly, we show that MiR-27a levels are significantly lower in patients with t(4;11) ALL than in t(12;21) patients (Figure 1B), thereby supporting the negative prognostic value of this miRNA in MLL-AF4-driven ALL.

We also show that MiR-27a plays an important role in the proliferation and survival of t(4;11) blasts because its ectopic expression in an MLL-AF4-positive ALL cell line significantly decreases the protein level of 14-3-3θ and of the transcription factor RUNX1 (Figure 1C,D), another MiR-27a target involved in this type of leukemia.[4,27] Indeed, MLL-AF4 transactivates the gene encoding RUNX1 that in turn dysregulates MLL-AF4 downstream genes, thereby contributing to the leukemic transcription program.[4,26,28,29]

A single miRNA is able to posttranscriptionally regulate the expression of multiple genes by targeting regulatory regions
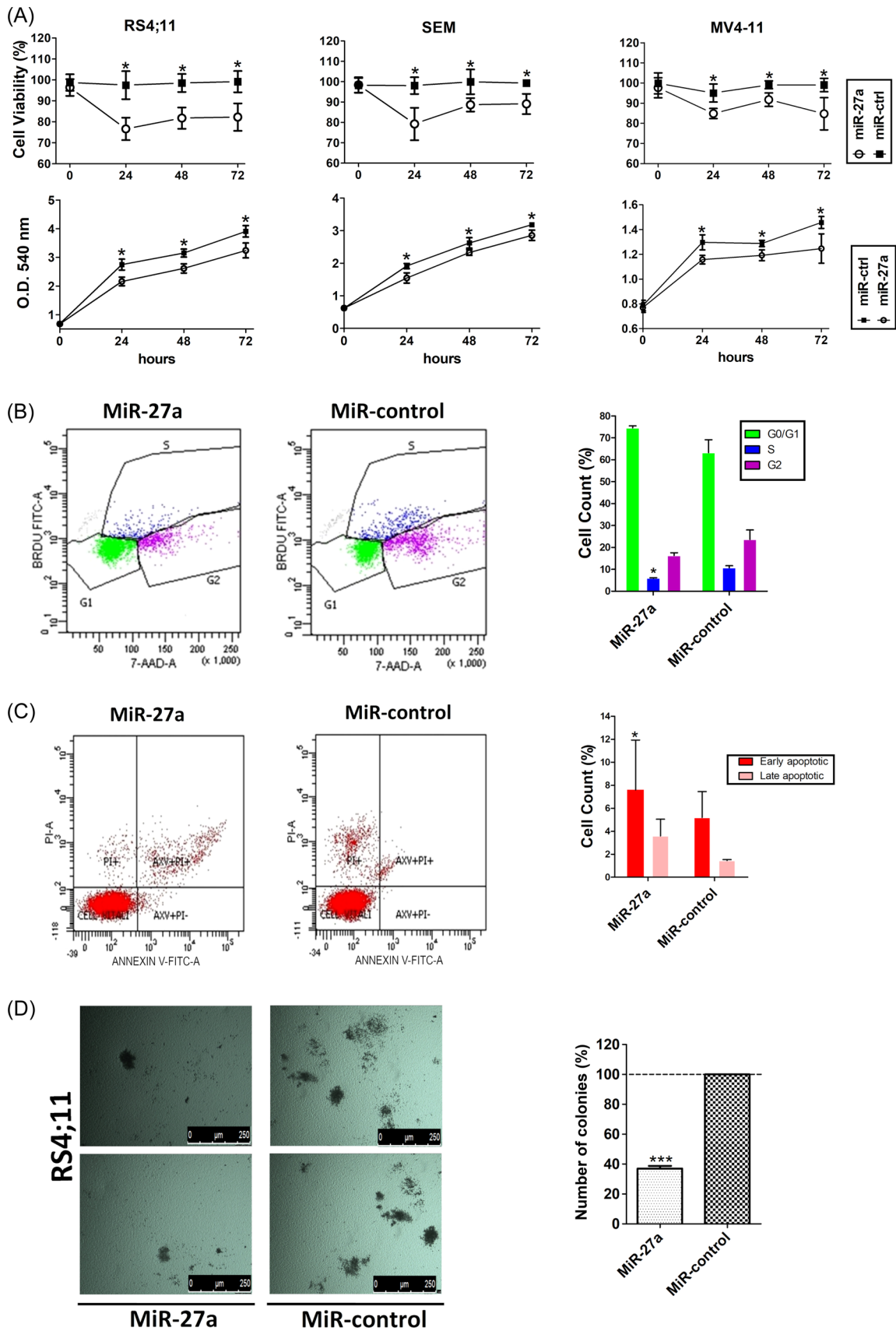
FIGURE 4 (See caption on next page)

within their 3′-UTR. We demonstrate that MiR-27a, besides 14-3-3θ and RUNX1, downregulates AF4 and MLL-AF4 expression (Figure 2A). Indeed, DLR assays demonstrate that 3′-UTR of AFF1 and KMT2A/AFF1 contain at least one seed sequence of MiR-27a. Accordingly, expression of a MiR-27a inhibitor in RS4;11 cells recovers protein levels of both AF4 and MLL-AF4 (Figure 2D). Intriguingly, AF4 and MLL-AF4, the master players in t(4;11) ALL molecular pathogenesis, are molecular partners of 14-3-3θ and RUNX1.[4,11,17,28]

As mutual interactors, 14-3-3θ, RUNX1, AF4, and MLL-AF4 take part in a protein complex that deregulates the expression of various genes, including the direct chimera targets.[28] Significantly, MiR-27a overexpression downregulates HOXA9, HOXA7, and MEIS1 expression, the main chimera targets, in all the tested t(4;11) cell lines (Figure 3A), whereas it does not affect the expression of these genes in REH, a non-MLL-related leukemia cell line (Figure 3A). Consistently, ChIP experiments reveal that MiR-27a overexpression actually decreases the binding of MLL-AF4 to HOXA9pr, whereas the binding of MLL WT is unaffected, in agreement with the target specificity of MiR-27a.

Consistently with previous studies demonstrating that HOXA9 knockdown hampers the proliferative potential and promotes apoptotic death of t(4;11) leukemia cells,[33] MiR-27a overexpression reduces viability and proliferative capability of t(4;11) leukemia cells (Figure 4A). Similar to other known miRNAs that target MLL-AF4 and AF4,[44–46] and with the antiapoptotic role of many protein members of the 14-3-3 family, including 14-3-3θ,[11,30,51] MiR-27a overexpression also increases the apoptotic rate of RS4;11 leukemia cells (Figure 4C). Since the blast's capability to form colonies depends on the potential of hematopoietic progenitors to differentiate and proliferate,[52] the significantly reduced clonogenic potential observed for RS4;11 leukemic blasts that overexpress MiR-27a (Figure 4D) is consistent with their reduced proliferative capability and increased apoptosis. As loss of differentiation and gain of uncontrolled proliferation are leukemic blast crucial features that are reversed, in t(4;11) cell lines, by ectopic expression of MiR-27a, our overall results strongly indicate that MiR-27a functions as a tumor suppressor in t(4;11) leukemia context.

In conclusion, we demonstrate that MiR-27a can negatively regulate four crucial drivers of blast transformation in t(4;11) leukemia pathogenesis, thereby emerging as a new potential target for the treatment of this still poorly curable and aggressive leukemia.

AUTHOR CONTRIBUTIONS

Tiziana Fioretti and Mariateresa Zanobio performed experiments, and prepared and analyzed figures. Maddalena Raia and Fabio Cattaneo performed partial experiments. Santa Errichiello and Barbara Izzo provided materials. Fabio Cattaneo and Rosario Ammendola revised the manuscript and the figures. Gabriella Esposito and Armando Cevenini conceived the project, supervised experiments, analyzed data, and wrote the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its Supporting Information: Materials.

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FIGURE 4   MiR-27a affects cell viability and proliferation of t(4:11) leukemic cells. (A) Viability of RS4;11, SEM, and MV4-11 leukemia cells at 24, 48, and 72 h after transfection with MiR-27a or MiR-negative control. Results are reported both as viability percentage and as cell growth increase expressed as spectrophotometric absorbance (O.D.) at 540 nm. (B) RS4;11 cell proliferation analyzed using BrdU assay, 48 h after cell transfection with MiR-27a or MiR-negative control. Results are represented as cytograms and bar graphs. (C) Cell apoptosis was determined by cytofluorimetric analysis of annexin-V-FITC (AXV) and propidium iodide (PI) staining at 72 h after cell transfection with MiR-27a or MiR negative control. Early apoptotic cells are AXV positive, and late apoptotic cells show AXV and PI staining. Results are graphically represented as cytograms and bar graphs. (D) Colony formation assay performed by culturing RS4;11 cells in a semisolid methylcellulose-based media at 24 h after transfection with MiR-27a or MiR-negative control. Pictures illustrate differences in size and number of colonies after 2 weeks in culture; the bar graph represents colony percentage (%). Error bars indicate the standard deviations. 7-AAD, 7-aminoactomycin D; BrdU, 5-bromo-2′-deoxyuridine; PI, propidium iodide. *$p < .05$; ***$p < .005$.

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