Leukemia Research 39 (2015) 479–485

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

microRNA-181a enhances cell proliferation in acute lymphoblastic leukemia by targeting EGR1

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ARTICLE INFO

Article history:
Received 4 August 2014
Received in revised form
19 December 2014
Accepted 20 January 2015
Available online 28 January 2015

Keywords:
Acute lymphoblastic leukemia (ALL)
miR-181a
EGR1
onco-miRNA
Cell proliferation
Cell cycle

ABSTRACT

Acute lymphoblastic leukemia (ALL) is an aggressive cancer that occurs in both children and adults. Starting from an integrated analysis of miRNA/mRNA expression profiles in 20 ALL patients, we identify a negative correlation between miR-181a and EGR1. Coherently, miR-181a over-expression in Jurkat T-ALL cells decreases EGR1 expression, increasing cell proliferation and enhancing the cell-cycle progression from G_1 to S phase. We show that EGR1 is a new direct target of miR-181a. Our findings suggest that miR-181a behaves as an onco-miRNA in ALL by down-regulating EGR1.

1. Introduction

Acute lymphoblastic leukemia (ALL) is a malignant neoplasm that affects both adults and children. It is the most common pediatric hematologic tumor, representing over a quarter of all pediatric cancers [1–3]. Several cytogenetic abnormalities have been identified as one of the causes of ALL, in particular associated with distinct immunologic phenotype and treatment outcome [4–6]. Moreover, it has been shown that also epigenetic factors, as DNA methylation and chromatin remodeling, are strongly involved in etiology of ALL [7,8]. Several studies have highlighted MicroRNAs (miRNAs) expression deregulation in ALL [9–11]. miRNA are a class of non-coding, single stranded RNAs composed of approximately 22 nucleotides, acting as negative regulators of gene expression at the post-transcriptional level. They are able to recognize their target mRNAs by imperfect base pairing with 3′-untranslated region (3′-UTR) [12]. The interaction between a miRNA and its target results in the inhibition of translation and/or destabilization of target mRNA molecules [13]. miR-181a is known to play an important regulatory role in leukocyte cell differentiation and function [14]. It has been shown that miR-181a has a key role in T-cell maturation. Particularly, miR-181a is specifically enriched at the $CD4^+CD8^+$ stage of thymocyte development, and it is able to inhibit the expression of genes involved in positive selection and T-cell maturation such as BCL2, CD69, and TCR [15]. miR-181a acts also as a "rheostat" during T-cell development, governing T-cell sensitivity and selection [16].

Furthermore, aberrant expression of miR-181a has been reported in several types of cancer, for instance in non-small-cell lung cancer [17], breast cancer [18], hepatocellular carcinoma [19], gastric and colon cancer [20,21], glioma [22] and oral squamous cell carcinoma [23].

In this work, we identify EGR1 as a miR-181a target. The early growth response gene 1 (EGR1) is a member of the early growth response (Egr) family of genes encoding for zinc-finger transcription factors [24]. EGR1 has a dual nature: it can act both as an oncogene or a tumor suppressor. It is oncogenic in prostate and gastric cancer [25,26], but it has tumor suppressor properties in different human tumors including non-small-cell lung cancer [27] and breast cancer [28]. It is known that EGR1 abrogates the E2F1 block in terminal myeloid differentiation suppressing its leukemia-promoting function. EGR1 promotes G0/G1 cell-cycle arrest behaving like a tumor suppressor gene [29].

EGR1 has been identified by us as a putative miR-181a target by two different approaches. First, we have used a novel analytical

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http://dx.doi.org/10.1016/j.leukres.2015.01.010

method to combine miRNA and gene expression profiles from a series of adult ALL patients [30], secondly, we analyzed the whole-transcript expression profiling of Jurkat cells (T-ALL cell line) over-expressing miR-181a using a microarray.

We demonstrate that miR-181a strongly reduces EGR1 expression, and enhances the proliferation of Jurkat T cells. Our study shows that miR-181a acts as an onco-miRNA in T-ALL cells regulating the expression of tumour suppressor gene EGR1. For the first time, EGR1 is shown to be a miR-181a target, opening new perspectives in cancer therapies.

2. Materials and methods

2.1. Cell and culture conditions

Jurkat cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and cultured in RPMI-1640 Medium (SIGMA) supplemented with 10% fetal bovine serum (SIGMA), at 37 °C in a 5% CO_2 atmosphere.

2.2. Construction of a lentiviral vector expressing miR-181a

PGK-miR181a expression construct was obtained amplifying a region of about 200 nt containing miR-181a precursor, from human genomic DNA. We used the following primers designed to incorporate *Rsr*I and *Nhe*I restriction sites and 6 bp of extra random sequence to aid in restriction digestion: 5′-GCGCGCCGGACCGAAGTGAGTAGAATTCTGAGTTTTGAGG-3′ (forward), 5′-GCGCGCGCTAGCTGTCTTCAGCGAATTCTGAGC-3′ (reverse). The amplified region was cloned into the *Rsr*I *Nhe*I sites of #1074.1071.hPGK.GFP.WPRE.mhCMV.dNGFR.SV40PA (kindly provided by Prof. Luigi Naldini). PGK-control was obtained by substituting miR-181a precursor fragment (*Rsr*I–*Nhe*I) with a sequence encoding for an hairpin yielding a 22-mer RNA designed to lack homology to any human gene. The lentiviral vector expressing miR-181a and the packing vectors pLP1, pLP2, and pLP/VSVG were co-transfected into HEK293T cells, and culture medium was collected at 48 h. The medium was filtered through 0.45-μm pore nitrocellulose filters and then centrifuged in a Beckman ultracentrifuge (Beckman, Optima™ LE-80K) at 25,000 rpm for 2 h at 4 °C. The precipitate was re-suspended in RPMI complete medium. The viral supernatant and Polybrene 8 μg/ml were added to the Jurkat cells.

2.3. Generation of Jurkat-miR-181a + EGR1 cell lines

The lentiviral vector expressing EGR1 (pLenti-GIII-UbC-Human-EGR1 Lentiviral Vector; abm) and the packing vectors pLP1, pLP2, pLP/VSVG were co-transfected into HEK293T cells, and culture medium was collected at 48 h. Blank vector (pLenti-UbC-Blank Vector; abm) was used as negative control in place of the vector expressing EGR1. The medium was filtered and centrifuged as described above. The precipitate was re-suspended in RPMI complete medium. The viral supernatant and Polybrene 8 μg/ml were added to the Jurkat-Control cell lines or Jurkat-miR-181a cell line.

2.4. Microarray data preprocessing

Three biological replicates for each sample were hybridized on the array (Human 1.0 ST microarrays, Affymetrix, Santa Clara, CA). Affymetrix. “CEL” files were separately processed for each replicate and experimental condition using the Robust Multi-array Average (RMA) procedure with quantile normalization, log2 transformation and background subtraction, as implemented in the Bioconductor package “oligo”. Then, as implemented in the R package “genefilter”, a generic filter was applied to the normalized expression values as follows: (I) remove Affymetrix control probesets; (II) remove probesets marked by low or null expression in total RNA samples (i.e.: require expression value > $\log_2(100)$ in both miR-181a over-expressing samples and control samples; (III) remove probe sets lacking annotation to a gene symbol. Therefore, we ended up with a list of 6300 filtered probesets. The “limma” package from BioConductor open source software for bioinformatics (under R statistical software) was used to calculate moderated t-statistics (based on the empirical Bayes approach) to identify differentially expressed genes between the miR-181 over-expressing group and control group. This led to the identification of differentially expressed genes between the two experimental conditions. Because of multiple hypothesis testing, p values were adjusted by the Benjamini-Hochberg false discovery rate (FDR) method. This led to the detection of a top list of two differentially expressed genes with and adjusted p-value under the 0.05 threshold.

2.5. RNA isolation, reverse transcription (RT), and quantitative real-time PCR (qPCR)

The total RNA was isolated from cells using Trizol (Invitrogen Life Technologies). Reverse transcription and quantitative real-time PCR of mature miR-181a and small nucleolar RNA U44 normalization control were performed as described [31,32] with minor changes. Briefly, 500 ng of total RNA was reverse transcripted by Enhanced Avian Reverse Transcriptase (Sigma), using the following primers: miR-181a RT primer 5′-AGGTCGTCGGTATTGCTGACTCACCGAC -3′; RNAU44 reverse primer 5′-GTCAGTTAGAGCTAATTAAGA-3′. Quantitative PCR (qPCR) was performed by SensiMix™ SYBR & Fluorescein Kit (Bioline). The level of mature miR-181a expression was normalized to those of internal control (RNAU44). Mature miRNAs were quantified with the standard curve method.

Oligonucleotides used for qPCR of human mature miRNA-181a and RNAU44 were: miR-181a forward 5′AA+A+T+AACGCTGTC-3′ (nucleotides preceded by ‘+’ are LNA modified), reverse 5′-AGGTCGTCGGTATTGCTG-3′; RNAU44 forward 5′-CCTGGATGATGATAAGCAATG-3′, reverse 5′-GTCAGTTAGAGCTAATTAAGA-3′. Abundance of EGR1 and EGR1 target genes mRNA were determined by RT-qPCR, using GAPDH as normalizing gene, according to the manufacturer’s instructions (SensiMix™ SYBR & Fluorescein Kit (Bioline)). Oligonucleotides used for RT-qPCR of human mRNA were: EGR1 forward 5′-AGCCCTACGAGCACCTGAC-3′, reverse 5′-GGTTTGGCTGGGGTAACTG-3′; TGFβ1 forward 5′-ACTACTACGCCAAGGAGGTCAC-3′, reverse 5′-TGCTTGAACTTGTCATAGATTTCG-3′; PTEN forward 5′-GCACAAGAGGCCCTAGATTTC-3′; reverse 5′-CGCCTCTGACTGGGAATAGT-3′; BCL2 forward 5′-AGTACCTGAACCGGCACCT-3′; reverse 5′-GCCGTACAGTTCCACAAAGG-3′; p53 forward 5′-GGGGTGACCTGGAGACCTAC-3′, reverse 5′-AGGAGAAGTGGGGCACCT-3′; p73 forward 5′-CTGGAGCTGATGGAGTTGG-3′, reverse 5′-ACGGGGGCTGTAGGTGAC-3′; Fibronectin forward 5′-CTGGCCGAAAATACATTGTAAA-3′, reverse 5′-CCACAGTCGGGTCAGGAG-3′; Gapdh: forward 5′-GAAATCCCATCACCATCTTCCAGG-3′, reverse 5′-GAGCCCCAGCCTTCTCCATG-3′.

2.6. Western blot analysis

Samples of 1×10^6 cells were washed with ice-cold PBS buffer and lysed with Ripa Lysis Buffer. Proteins (30 μg/lane) were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblots were probed with the following primary antibodies: anti-EGR1 (1:500 Cell Signaling), and anti-GAPDH (1:20,000 Cell Signaling). Signals were revealed after incubation with recommended secondary antibody coupled to peroxidase using chemiluminescent HRP substrate reagent (Thermo Scientific).

2.7. Construction of plasmid EGR1_3′-UTR

Human genomic DNA was isolated and purified from Jurkat with DNesy® Blood and Tissue Kit according to the manufacturer's protocol. The 3′-untranslated region (UTR) sequence of EGR1 was PCR amplified from the genomic DNA using the following primers: forward 5′-GCGCGCTCTAGACTTCCCTTCCTCAGCTGTCA-3′ and reverse 5′-GCGCGCTCTAGATATCCCATGGGCAATAAAGC-3′. The primers were designed incorporating *Xba*I restriction site and 4 bp of extra random sequence to aid in restriction digestion. *Xba*I-digested PCR products were cloned into a *Xba*I-digested pGL3 dual luciferase reporter vector (Promega). The mutated version EGR1_3′-UTR mut, was generated utilizing the EGR1_3′-UTR plasmid as template and modifying the miR-181a seed binding site using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies).

The mutagenic primers used were: forward 5′-TTATAAATATATTCAGGAGTTGGTTGTAGTTACCTACTGAGTAGGC-3′ and reverse 5′-GCCTACTCAGTAGGTAACTACAACCAACTCCTGAATATATTTATAA-3′.

2.8. Dual luciferase assays

For luciferase assays, HEK293T cells were seeded onto 24-well plate (1×10^6 cells each well) the day before transfection. 300 ng of pGL3-firefly-EGR1_3′-UTR and 300 ng of pCMV-miR-181a (ORIGENE) or pCMV-control were co-transfected into HEK293T cells. Ten ng of pRL-TKRenilla were included as internal control. The same assay was performed using pGL3-firefly-EGR1_3′-UTR-mut instead of pGL3-firefly-EGR1_3′-UTR. Forty-eight hours later, luciferase activity was detected by Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized to Renilla activity for each transfected well.

2.9. Cell proliferation assay

Cell proliferation was determined by cell counting and CellTiter 96 AQueous assay (Promega). In cell counting, 10^5 cells were seeded in 12-well plates in duplicate and grown for 72 h. Cell counting was performed after 24, 48 and 72 h. Cell titer 96 AQueous assay was performed according to the manufacturer's recommendations.

2.10. Cell-cycle analysis

10^6 cells were fixed in cold ethanol, washed, resuspended in phosphate-buffered saline containing 50 μg/ml propidium iodide (PI) and 50 μg/ml RNAase A, and analyzed by flow cytometry on a FACScan (Becton Dickinson).

2.11. Apoptotic assay

10^4 cells were seeded in 96-well plates in triplicate and apoptotic cells were determined using the Caspase-Glo3/7 assay system (Promega) according to the manufacturer's recommendations.

2.12. Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical differences were determined by paired *t* test, with values of $p < 0.05$ considered statistically significant. Results were expressed as mean ± S.E.M. of at least three independent experiments.

3. Results

3.1. Identification of putative targets by integrated analysis of miRNA and mRNA expression profiles

The involvement of different miRNAs in a series of adult ALL cases was explored in our previous work [30] using a novel analytical method to combine miRNA and gene expression profiles. We identified miRNA/mRNA pairs with positive and negative expression correlation as defined in [30]. Focusing our attention on pairs displaying the lowest negative correlation scores, we decided to investigate the nature of the correlation between miR-181a and EGR1. Fig. 1A shows the negative correlation between the miR-181a expression and the expression of its putative target EGR1 in each of the 20 ALL cases analyzed (Pearson correlation (r) = −0.66).

3.2. Identification of miR-181a targets by gene expression analysis

Jurkat cells, a well-known immortalized line of human T-ALL, have been used as *in vitro* model in our study. Since miR-181a expression level in Jurkat cells is quite low (data not shown), they

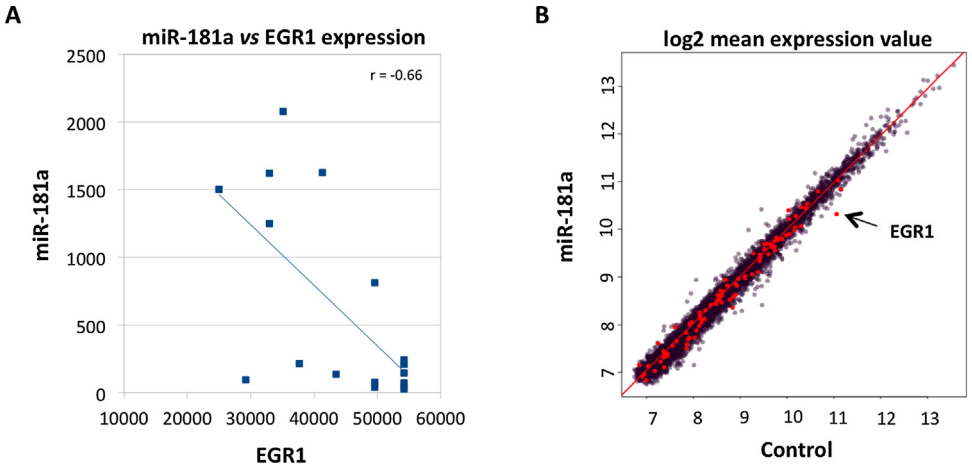

Fig. 1. Identification of putative miR-181a targets. (A) The graph shows the negative correlation between miR-181a and its putative target EGR1 in each of 20 ALL cases analyzed (Pearson correlation (r) = −0.66). (B) The graph displays genes expression modulation in response to miR-181a over-expression, in three independent biological replicates. EGR1 is indicated by the black arrow.

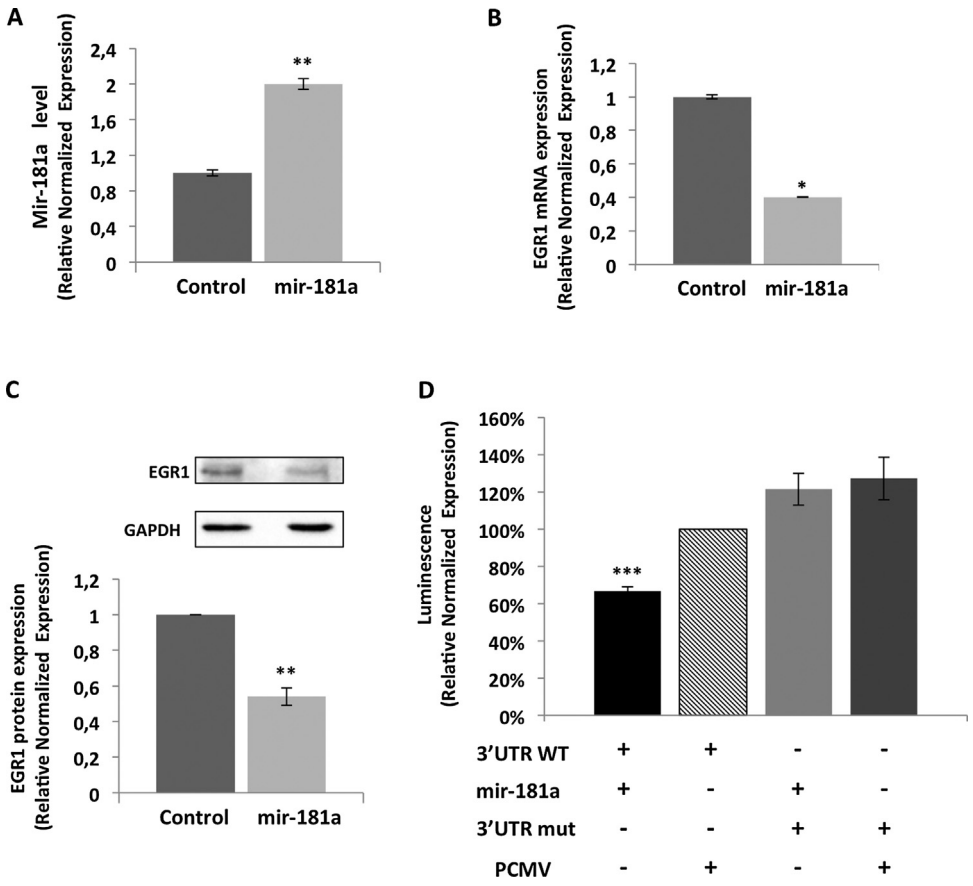

Fig. 2. miR-181a down-regulates EGR1 by directly binding its 3′-UTR**.** miR-181a (A) and EGR1 mRNA expression (B) were detected by RT-qPCR in Jurkat-miR-181a cell line and in Jurkat-Control cell line. Overexpression of miR-181a significantly down-regulates mRNA expression of EGR1. (C) Western blot assays show that overexpression of miR-181a reduces the EGR1 protein expression. GAPDH was used as internal loading control. *Top*, a representative of the Western blot. *Bottom*, densitometric quantification of three independent experiments performed as described above. Band intensity was normalized to that of GAPDH. (D) Dual luciferase assays show that miR-181a reduces luciferase activity in cells containing the EGR1_3′-UTR. Relative luciferase activities of EGR1_3′-UTR and mutant (mut) 3′-UTR regions were obtained by co-transfection of PCMV-miR-181a precursor or control plasmid PCMV. The negative control (EGR1_3′-UTR with PCMV) was set as 100%. Firefly activity was normalized to that of Renilla. The results in all figures were presented as mean ± SEM from three independent experiments (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (paired *t*-test)).

represent a good cell model to over-express miR-181a and to analyze the resulting phenotype. For this purpose, we generated a miR-181a lentiviral-based expression vector (pRRL-miR-181a) and transduced it in Jurkat cells. To assess the specificity of miR-181a effects, we used as control a lentiviral construct (pRRl-ct), encoding for a hairpin RNA designed to lack homology to any human gene. After performing lentiviral infections, the over-expression of mature miR-181a has been confirmed by mature miRNA-specific RT-qPCR. Fig. 2A shows that overexpression of miR-181a results in a 2-fold increase in Jurkat-miR-181a cell lines compared to Jurkat-Control cell lines (Fig. 2A). After the generation of the Jurkat-miR-181a cell line, we compared the mRNA expression profile of miR-181a overexpressing cell line with the one of the control cell line, in three independent biological replicas. In particular, the Gene Chip Human Gene 2.0 ST Array (Affimetrix) has been used. The scatter plot in Fig. 1B shows the genes modulated in consequence of miR-181a over-expression. Among the most statistically significant modulated genes, we focused on miR-181a putative target genes predicted by two different microRNA databases such as TargetScan and miRanda. EGR1 has been found to be the most modulated target of miR-181a, as indicated in Fig. 1B. These results are in line with our original analysis [30] suggesting EGR1 as a miR-181a target.

3.3. *EGR1 is a target of miR-181a*

In order to confirm that EGR1 is a target of miR-181a, we evaluated the expression level of EGR1 mRNA by RT-qPCR and of EGR1 protein by western blot analysis in Jurkat-miR-181a cell lines versus Jurkat-Control cell lines. EGR1 mRNA and EGR1 protein level were decreased by about 60% (Fig. 2B) and 50% (Fig. 2C), respectively. To confirm that miR-181a is a direct regulator of EGR1, the 3′-UTR of human EGR1 (EGR1_3′-UTR) was cloned downstream of a luciferase coding sequence. We performed luciferase assays in HEK293T cell line, comparing the luciferase activity of EGR1_3′-UTR co-transfected with a plasmid containing mir-181a precursor (PCMV-miR-181a), against the luciferase activity of EGR1_3′-UTR co-transfected with a control plasmid PCMV. Co-transfection with PCMV-miR-181a causes a 33% reduction in EGR1_3′-UTR luciferase expression (Fig. 2D). To further demonstrate the miRNA-target gene direct interaction, we performed a site-specific mutagenesis in the putative miRNA binding site of the EGR1_3′-UTR sequence, and then repeated the luciferase reporter assay. Inactivation of miR-181a binding sites in the EGR1_3′-UTR restored luciferase expression, indicating a direct binding of miR-181a on EGR1_3′-UTR (Fig. 2D).

3.4. *Biological consequences of miR-181a over-expression in T-acute lymphoblastic leukemia cells*

Given the importance of EGR-1 in the regulation of cell-cycle progression [29], we addressed the role of miR-181a in Jurkat cell proliferation. Overexpression of miR-181a by lentiviral infection significantly up-regulated the proliferation rate of Jurkat cells detected by cell counting and also the cell viability detected by MTS tetrazolium assay (Fig. 3A and B). To better understand the

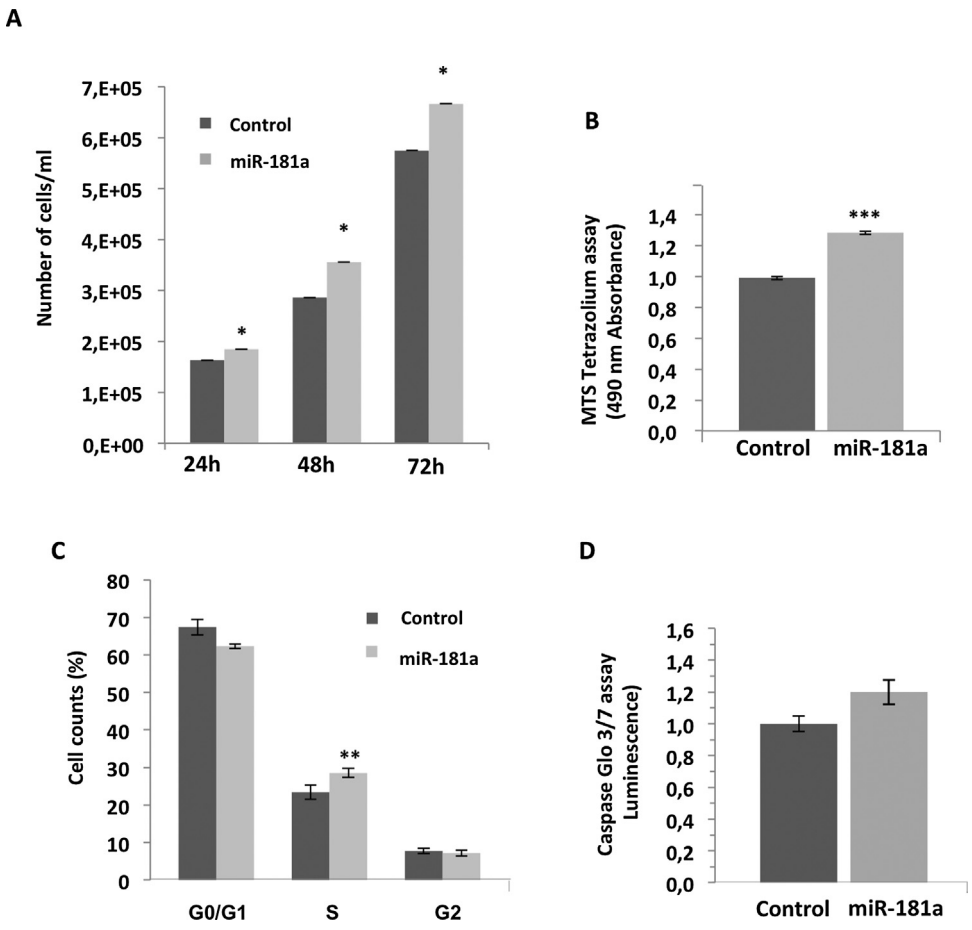

Fig. 3. Overexpression of miR-181a in Jurkat T-cell increases cell proliferation and promotes cell-cycle progression. Cell proliferation and cell viability of Jurkat miR-181a and Jurkat Control cell line were detected by cell counting after 24, 48 and 72 h from seeding (A) and by MTS tetrazolium assay after 48 h from seeding, respectively (B). Both assays were repeated five times on independent cell cultures. (C) Propidium iodide flow cytometric assay was performed to analyze the cell cycle. In Jurkat miR-181a cell line we observed an increase of S phase and a decrease of G0/G1 phase in comparison to Jurkat control cell line. Cell-cycle analysis was performed in triplicate. (D) Caspase Glo 3/7 luminescent assay was used to detect caspase-3/7 activities. We observed no difference between Jurkat miR-181a and Jurkat Control cell line. Assay was performed six times. The results in all figures were presented as mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (paired t-test)).

regulation of Jurkat cell proliferation by miR-181a, we examined cell-cycle profile. Jurkat-miR-181a cells displayed a significant increase in the number of cells in S phase and a decrease in the number of cells in G_0/G_1 phase (Fig. 3C). This indicates that overexpression of miR-181a enhances cell-cycle progression by promoting G_1 to S phase transition. We also tested the apoptotic rate in Jurkat-miR-181a measuring Caspase3/7 activities. We did not observe any statistically significant difference between apoptosis level in Jurkat-miR-181a cell line compared to Jurkat-Control cell line (Fig. 3D).

3.5. *Biological effects of miR-181a over-expression are mediated by EGR1*

In order to verify that the phenotype associated with miR-181a over-expression in Jurkat cell is mediated by EGR1, we evaluated by RT-qPCR the expression level of different EGR1 targets genes involved in cell proliferation and cell cycle [33]. In particular, we measured the expression of TGFβ1, BCL2, p53, p73, PTEN and Fibronectin [33,35–40]. Coherently with our hypothesis, four out of six genes (TGFβ1, BCL2, p53 and p73) show a significant reduction of expression in miR-181a overexpressing cells. The PTEN expression is reduced even though its change does not attain statistical significance, while no change is observed in the Fibronectin expression (Fig. 4A).

To provide additional evidences that the biological effects of miR-181a is mediated by EGR1, we went on restoring EGR1 expression in Jurkat-miR-181a by lentiviral-based expression vector (pLenti-GIII-UbC-Human-EGR1 Vector) and observing the resulting phenotype. To assess the specificity of EGR1 effects, we used as control a lentiviral construct indicated as blank (pLenti-UbC-Blank Vector) and transduced it in Jurkat-miR-181a and Jurkat-Control cell lines. We obtained the three cell lines Jurkat-miR-181a + EGR1, Jurkat-miR-181a + blank and Jurkat-Control + blank.

The rescue of EGR1 protein in miR-181a over-expressing cells was confirmed by western blot analysis (Fig. 4B). The cell proliferation profile of Jurkat-miR-181a + EGR1 is similar to those of Jurkat-Control + blank cell line (Fig. 4C and D). In detail, the cell viability of Jurkat-miR-181a + EGR1 cell line is significantly reduced in comparison to Jurkat-miR-181a + blank and it is similar to those of Jurkat-Control + blank cell line (Fig. 4C). In a coherent manner, in the cell-cycle analysis the percentage of cells in S-phase in Jurkat-miR-181a + EGR1 is significantly reduced when compared to Jurkat-miR-181a + blank cell line. These evidences show that the rescue of EGR1 protein is able to revert the phenotype originally observed in Jurkat-miR-181a cell line, demonstrating that EGR1 decrease has an essential role in defining the biological effects of miR-181a over-expression.

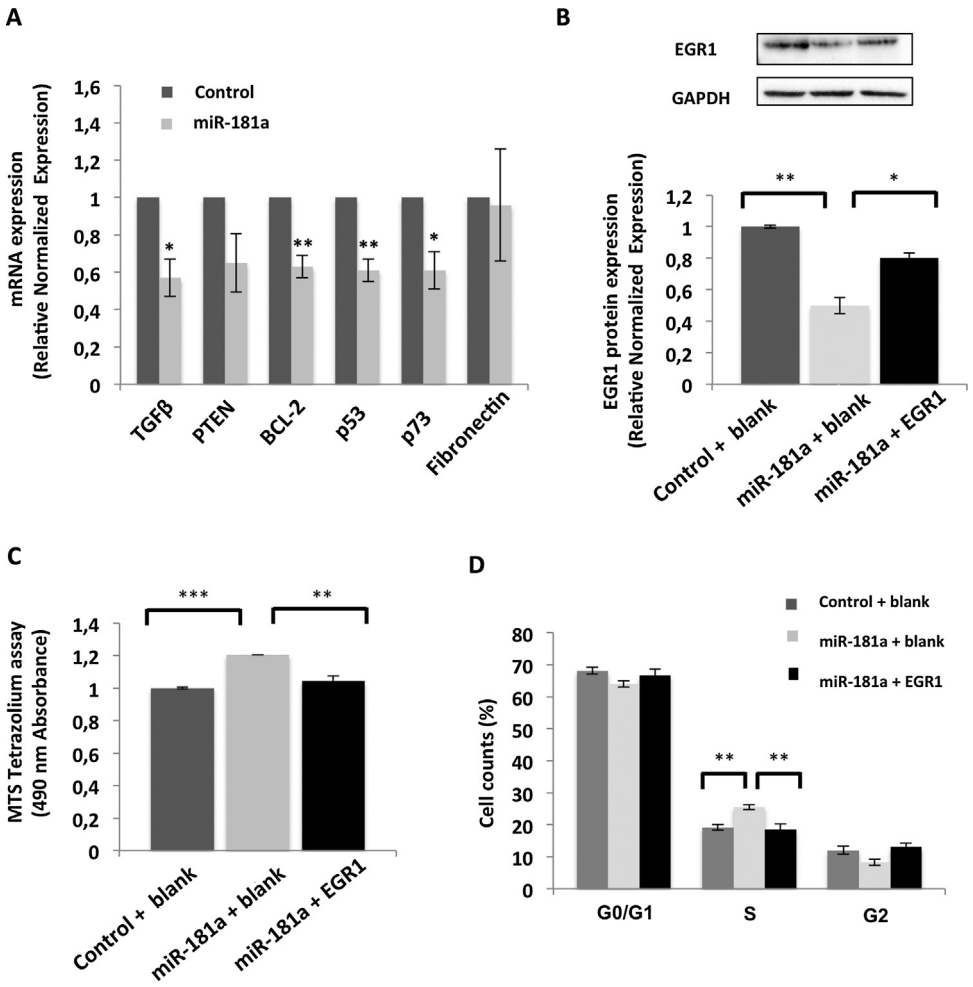

Fig. 4. The phenotype of miR-181a over-expression is mediated by EGR1. (A) TGFβ1, BCL2, p53, p73, PTEN and Fibronectin mRNA expression were detected by RT-qPCR in Jurkat-miR-181a cell line and in Jurkat-Control cell line. (B) Western blot assays show the rescue of EGR1 protein in miR-181a over-expressing cells. GAPDH was used as internal loading control. *Top*, a representative of the Western blot. *Bottom*, densitometric quantification of three independent experiments performed as described above. Band intensity was normalized to that of GAPDH. (C) Cell viability of Jurkat Control + blank, Jurkat miR-181a + blank and Jurkat-miR-181 + EGR1 cell line was detected by MTS tetrazolium assay after 48 h from seeding. The assay was repeated five times on independent cell cultures. (D) Propidium iodide flow cytometric assay was performed to analyze the cell cycle. Cell-cycle analysis was performed six times. The results in all figures were presented as mean ± SEM (*$p < 0.05$;**$p < 0.01$; ***$p < 0.001$ (paired *t*-test)).

4. Discussion

This work was inspired by the expression analysis of miRNA and mRNA profiles in 20 ALL patients. In order to understand the functional regulatory effects of differential miRNAs on their targets, we used a novel analytical method to combine their expression profiles [30]. From this analysis, we selected the pair miR-181a/EGR1, whose negative correlation was confirmed thereafter by *in vitro* experiments using Jurkat T-ALL cell line. This cell line is notoriously difficult to transfect transiently; therefore, we performed a lentiviral infection to obtain a stable Jurkat cell line over-expressing miR-181a. By gene expression analysis we obtained a list of genes modulated as a consequence of miR-181a over-expression. Among these genes we focused on the miR-181a targets, finding EGR1 as the most statistically significant modulated one. The phenotype of miR-181a over-expressing cells is characterized by the increase of cell viability and proliferation, and by the promotion of the cell-cycle progression.

The same miRNA can have functions either as tumor suppressor or oncogene, depending on the cellular context and on the consequent expression of its targets [34]. An example of miRNA that has such dual behavior is the miR-181a. It is known that miR-181a acts as a tumor suppressor in glioma [22] and in oral squamous cell carcinoma [23], but it behaves as an onco-miRNA in non-small-cell lung cancer [17], breast cancer [18], hepatocellular carcinoma [19] and gastric and colon cancer [20,21]. In agreement with the latter examples, we observed that miR-181a has a role of onco-miRNA in ALL.

In this work, we show that EGR1 is a new direct target of miR-181a. Our data suggest that EGR1 is responsible, at least in part, for the phenotype observed in Jurkat over-expressing miR-181a. There are instances of the EGR1 activity as oncogenes [25,26], but also other instances where it has tumor-suppressive properties [27–29]. In agreement with the EGR1 role in non-small-cell lung cancer [27], breast cancer [28] and myeloid leukemia [29], our data support the idea that EGR1 has a role of tumor suppressor in ALL. Indeed, this is demonstrated by the analysis of expression conducted on some genes directly or indirectly subject to EGR1 regulation. In particular, we focused on TGFβ1, BCL2, p53 and p73, whose transcription is promoted by EGR1 [35–40]. Analyzing the expression of such genes, we observed a reduction in miR-181a over-expressing cells when compared to Jurkat-Control cell line.

To test directly the EGR1 capability of acting as tumor suppressor gene in ALL, we attempted to produce a stable Jurkat cell line down-expressing EGR1. Unfortunately, the Jurkat cells fail to survive when the expression level of EGR1 is dramatically reduced (data not shown).

On the contrary, Jurkat-miR-181a cells are able to survive and proliferate when we partially restore the EGR1 expression. Jurkat-miR-181a + EGR1 cells show a reduction of cell viability and a decrease of cells in S-phase in comparison to Jurkat-miR-181a + blank.

The rescue of EGR1 leads to a phenotype similar to the one of the control cells, demonstrating that EGR1 is responsible for the phenotype observed in Jurkat over-expressing miR-181a.

In conclusion, we obtained evidences that miR-181a can act as an onco-miRNA repressing the tumor suppressor EGR1. Our work opens the way for a series of future studies in which the reciprocal expression of miR-181a and its target EGR1 may be useful in predicting clinical outcome in ALL. Although other studies are necessary to thoroughly elucidate the role of the miR-181a/EGR1 interplay, the discovery of their functional relationship will lead to a better understanding of molecular pathways involved in ALL, contributing to open new possibilities for future diagnosis, prognosis and therapies in patients affected by acute leukemia.

Funding

This work was supported by grants from AIRC IG 2010N.10085: "Characterization of the role of miR-103, let-7, miR-181, miR-19 and miR-93 in Acute Lymphoblastic Leukemias." and by FIRB grant "Nuovi marcatori per valutare suscettibilità, prognosi e possibilità di terapie mirate nelle leucemie acute, *de novo* e secondarie del bambino e dell' adulto".

We thank Dr. Luigi Naldini for kindly providing published lentiviral constructs.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgements

Author's contributions: L.V., V.F., C.C. conceived and designed the experiments; L.V., G.A., I.L. performed experiment; S.G. performed bioinformatics analysis; L.V., V.F. wrote the paper; G.M. supervised the study.

References

[1] Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. N Engl J Med 2006;354(2):166–78.
[2] Vitale A, Guarini A, Chiaretti S, Foà R. The changing scene of adult acute lymphoblastic leukemia. Curr Opin Oncol 2006;18(6):652–9.
[3] Pui CH. Recent research advances in childhood acute lymphoblastic leukemia. J Formos Med Assoc 2010;109(11):777–87.
[4] Faderl S, Kantarjian HM, Talpaz M, Estrov Z. Clinical significance of cytogenetic abnormalities in adult acute lymphoblastic leukemia. Blood 1998;91(11):3995.
[5] Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N Engl J Med 2004;350(15):1535.
[6] Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. Lancet 2008;371(9617):1030.
[7] Nordlund J, Bäcklin CL, Wahlberg P, Busche S, Berglund EC, Eloranta ML, et al. Genome-wide signatures of differential DNA methylation in pediatric acute lymphoblastic leukemia. Genome Biol 2013;14(9):r105.
[8] Shi J, Whyte WA, Zepeda-Mendoza CJ, Milazzo JP, Shen C, Roe JS, et al. Role of SWI/SNF in acute leukemia maintenance and enhancer-mediated Myc regulation. Genes Dev 2013;27(24):2648–62.
[9] Li XJ, Luo XQ, Han BW, Duan FT, Wei PP, Chen YQ. MicroRNA-100/99a, deregulated in acute lymphoblastic leukaemia, suppress proliferation and promote apoptosis by regulating the FKBP51 and IGF1R/mTOR signalling pathways. Br J Cancer 2013;109(8):2189–98.
[10] Yan J, Jiang N, Huang G, Tay JL, Lin B, Bi C, et al. Deregulated MIR335 that targets MAPK1 is implicated in poor outcome of paediatric acute lymphoblastic leukaemia. Br J Haematol 2013;163(1):93–103.
[11] Burke MJ, Bhatla T. Epigenetic modifications in pediatric acute lymphoblastic leukemia. Front Pediatr 2014;2:42.
[12] Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120(1):15–20.
[13] Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008;9(2):102–14.
[14] Sun X, Sit A, Feinberg MW. Role of miR-181 family in regulating vascular inflammation and immunity. Trends Cardiovasc Med 2014;24(3):105–12.
[15] Neilson JR, Zheng GX, Burge CB, Sharp PA. Dynamic regulation of miRNA expression in ordered stages of cellular development. Genes Dev 2007;21(5):578–89.
[16] Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, et al. miR-181a is an intrinsic modulator of T cell sensitivity and selection. Cell 2007;129(1):147–61.
[17] Gao W, Yu Y, Cao H, Shen H, Li X, Pan S, et al. Deregulated expression of miR-21, miR-143 and miR-181a in non small cell lung cancer is related to clinicopathologic characteristics or patient prognosis. Biomed Pharmacother 2010;64(6):399–408.
[18] Taylor MA, Sossey-Alaoui K, Thompson CL, Danielpour D, Schiemann WP. TGF-β upregulates miR-181a expression to promote breast cancer metastasis. J Clin Invest 2013;123(1):150–63.
[19] Meng F, Glaser SS, Francis H, DeMorrow S, Han Y, Passarini JD, et al. Functional analysis of microRNAs in human hepatocellular cancer stem cells. J Cell Mol Med 2012;16(1):160–73.
[20] Zhang X, Nie Y, Du Y, Cao J, Shen B, Li Y. MicroRNA-181a promotes gastric cancer by negatively regulating tumor suppressor KLF6. Tumour Biol 2012;33(5):1589–97.
[21] Wei Z, Cui L, Mei Z, Liu M, Zhang D. miR-181a mediates metabolic shift in colon cancer cells via the PTEN/AKT pathway. FEBS Lett 2014;588(9):1773–9.
[22] Shi L, Cheng Z, Zhang J, Li R, Zhao P, Fu Z, et al. hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells. Brain Res 2008;1236:185–93.
[23] Shin KH, Bae SD, Hong HS, Kim RH, Kang MK, Park NH. miR-181a shows tumor suppressive effect against oral squamous cell carcinoma cells by downregulating K-ras. Biochem Biophys Res Commun 2011;404(4):896–902.
[24] Sukhatme VP, Kartha S, Toback FG, Taub R, Hoover RG, Tsai-Morris CH. A novel early growth response gene rapidly induced by fibroblast, epithelial cell and lymphocyte mitogens. Oncogene Res 1987;1(4):343–55.
[25] Zheng L, Pu J, Jiang G, Weng M, He J, Mei H, et al. Abnormal expression of early growth response 1 in gastric cancer: association with tumor invasion, metastasis and heparanase transcription. Pathol Int 2010 Apr;60(4):268–77.
[26] Virolle T, Krones-Herzig A, Baron V, De Gregorio G, Adamson ED, Mercola D. Egr1 promotes growth and survival of prostate cancer cells Identification of novel Egr1 target genes. J Biol Chem 2003;278(14):11802–10.
[27] Ferraro B, Bepler G, Sharma S, Cantor A, Haura EB. EGR1 predicts PTEN and survival in patients with non-small-cell lung cancer. J Clin Oncol 2005;23(9):1921–6.
[28] Liu J, Liu YG, Huang R, Yao C, Li S, Yang W, et al. Concurrent down-regulation of Egr-1 and gelsolin in the majority of human breast cancer cells. Cancer Genomics Proteomics 2007;4(6):377–85.
[29] Gibbs JD, Liebermann DA, Hoffman B. Egr-1 abrogates the E2F-1 block in terminal myeloid differentiation and suppresses leukemia. Oncogene 2008;27(1):98–106.
[30] Fulci V, Colombo T, Chiaretti S, Messina M, Citarella F, Tavolaro S, et al. Characterization of B- and T-lineage acute lymphoblastic leukemia by integrated analysis of MicroRNA and mRNA expression profiles. Genes Chrom Cancer 2009;48(12):1069–82.
[31] Raymond CK, Roberts BS, Garrett-Engele P, Lim LP, Johnson JM. Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. RNA 2005;11(11):1737–44.
[32] Fulci V, Chiaretti S, Goldoni M, Azzalin G, Carucci N, Tavolaro S, et al. Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. Blood 2007;109(11):4944–51.
[33] Baron V, Adamson ED, Calogero A, Ragona G, Mercola D. The transcription factor Egr1 is a direct regulator of multiple tumor suppressors including TGFβ1, PTEN, p53 and Fibronectin,. Cancer Gene Ther 2006;13(2):115–24.
[34] Shenouda SK, Alahari SK. MicroRNA function in cancer: oncogene or a tumor suppressor? Cancer Metastasis Rev 2009;28(3–4):369–78.
[35] Liu C, Adamson E, Mercola D. Transcription factor EGR-1 suppresses the growth and transformation of human HT-1080 fibrosarcoma cells by induction of transforming growth factor beta 1. Proc Natl Acad Sci USA 1996;93:11831–6.
[36] Huang RP, Fan Y, deBelle I, Ni Z, Matheny W, Adamson ED. Egr-1 inhibits apoptosis during the UV response: correlation of cell survival with Egr-1 phosphorylation. Cell Death Differ 1998;5:96–106.
[37] de Belle I, Huang RP, Fan Y, Liu C, Mercola D, Adamson ED. p53 and Egr-1 additively suppress transformed growth in HT1080 cells but Egr-1 counteracts p53-dependent apoptosis. Oncogene 1999;18:3633–42.
[38] Krones-Herzig A, Eileen A, Dan M. Early growth response 1 protein, a novel upstream gatekeeper of the p53 tumor suppressor, controls replicative senescence. Proc Natl Acad Sci USA 2003;100:3233–8.
[39] Krones-Herzig A, Mittal S, Kelly Y, Hongyan L, Chris E, Rafael U, et al. Early growth response 1 acts as a tumor suppressor in vivo and in vitro via regulation of p53. Cancer Res 2005;65(12):5133–43.
[40] Pignatelli M, Luna-Medina R, Pérez-Rendón A, Santos A, Perez-Castillo A. The transcription factor early growth response factor-1 (EGR-1) promotes apoptosis of neuroblastoma cells. Biochem J 2003;373:739–46.