MOLECULAR CANCER

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The tumor suppressive role of miRNA-370 by targeting FoxM1 in acute myeloid leukemia

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Abstract

Background: Recent evidence has accumulated that MicroRNA (miRNA) dysregulation occurs in the majority of human malignancies including acute myeloid leukemia (AML) and may contribute to onco-/leukemo-genesis.

Methods: The expression levels of miR-370 and FoxM1 were assessed in 48 newly diagnosed AML patients, 40 AML patients in 1st complete remission (CR) and 21 healthy controls. Quantitative real-time PCR, western blots, colony formation assay, and β-Galactosidase (SA-β-Gal) staining were used to characterize the changes induced by overexpression or inhibition of miR-370 or FoxM1.

Results: We found that the down-regulation of miR-370 expression was a frequent event in both leukemia cell lines and primary leukemic cells from patients with de novo AML. Lower levels of miR-370 expression were found in 37 of 48 leukemic samples from AML patients compared to those in bone marrow cells derived from healthy adult individuals. Ectopic expression of miR-370 in HL60 and K562 cells led to cell growth arrest and senescence. In contrast, depletion of miR-370 expression using RNA interference enhanced the proliferation of those leukemic cells. Mechanistically, miR-370 targets the transcription factor FoxM1, a well established oncogenic factor promoting cell cycle progression. Moreover, when HL60 and K562 cells were treated with 5-aza-2'-deoxycytidine, a DNA methylation inhibitor, miR-370 expression was up-regulated, which indicates epigenetic silencing of miR-370 in leukemic cells.

Conclusions: Taken together, miR-370 may function as a tumor suppressor by targeting FoxM1, and the epigenetic silence of miR-370 thus leads to derepression of FoxM1 expression and consequently contributes to AML development and progression.

Keywords: miR-370, FoxM1, AML, Cellular senescence

Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of neoplastic haematopoietic diseases characterized by proliferation and maturation arrest of myeloid blasts in bone marrow and blood [1]. The long-term overall survival (OS) rate for AML patients under the age of 60 years and 60 years or older is 30–40% and under 10%, respectively [2], which remains a challenge. Thus, it is urgently needed to search for new targets for molecularly designed therapies.

microRNAs (miRNAs), small (~22 nucleotide), single-stranded noncoding RNAs, are a novel class of biological molecules. Their genes may either give rise to single miRNAs, or contain several miRNAs in one transcriptional unit as miRNA clusters [3]. miRNAs post-transcriptionally repress gene expression by recognizing complementary target sites in the 3'untranslated region (UTR) of target mRNAs [4,5]. miRNAs have been implicated in a large variety of biological processes, including cell cycle progression, apoptosis, differentiation and haematopoiesis [6-10], and thereby play important roles in many pathological processes, including malignant transformation [11,12]. More than 50% of miRNA genes are located in cancer-associated genomic regions or in fragile sites, and both oncogenic and tumor suppressive

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functions have thus far been ascribed to specific miR-NAs [13]. Moreover, miRNAs have emerged as critical regulators of hematopoiesis and their aberrant expression has been associated with the pathogenesis of leukemia [14]. Functional validation of deregulated miR-NAs in hematopoiesis has been shown for several miR-NAs [15]. Distinctive patterns of increased expression and/or silencing of multiple miRNAs have been associated with specific cytogenetic and molecular subsets of AML [16]. miR-370 has been noted to be down-regulated in papillary thyroid carcinoma, colorectal cancer [17] and malignant cholangiocytes [18], but evidence of a biological role for this miRNA in AML has not been reported. In the present study, we sought to define the role of miR-370 in AML by investigating its expression and biological function in leukemic cell lines and blast cells from patients with de novo AML.

Results

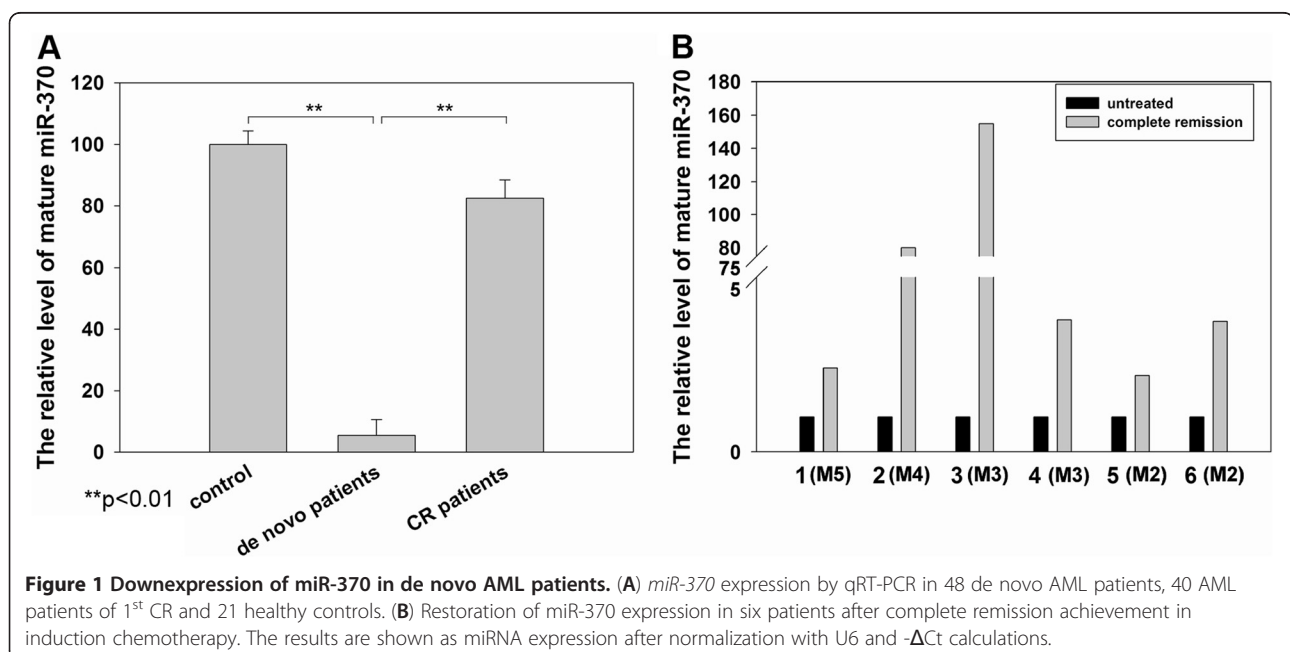### Down-regulation of miR-370 expression in BM blasts from de novo AML patients

We analyzed miR-370 expression in BM samples from 48 de novo AML patients at diagnosis time using qRT-PCR. As shown in Figure 1A, the miR-370 level in patients' samples was significantly reduced ($P < 0.01$, t test) compared to that from healthy controls, while following acquisition of CR in the induction chemotherapy, miR-370 expression level restored to 0.82 fold of controls. There was no association between the presence of mature miR-370 and age, gender, blast percentage or FAB subtypes (data not shown). In six patients, BM samples were available both at diagnosis time prior to

treatment and after a complete remission and we found a lower miR-370 level at diagnosis while at least 2.1-fold increase in miR-370 expression after CR (Figure 1B).

Changes in proliferation and cellular senescence of leukemic cells mediated by altered miR-370 expression

We then explored the biological function of miR-370 in leukemic cells. Cells were transfected with precursors to miR-370 and miR-370 inhibitor to enhance and decrease mature miR-370 expression, respectively. Transfection with the miR-370 precursor increased mature miR-370 expression 114.5 ± 5.70: 1 ± 0.12 ($p < 0.05$) and 59.8 ± 6.90: 1 ± 0.24 ($p < 0.05$) (pSilencer-miR vs pSilencer) times higher in HL60 and K562 cells, respectively (Figure 2A). Overexpression of miR-370 decreased cell proliferation (Figure 2C and Additional file 1) (pSilencer vs pSilencer-miR: HL60: 88 ± 15 vs 11 ± 4, $p < 0.01$; K562: 49 ± 5 vs 18 ± 5, $p < 0.01$). On the other hand, transfection with the miR-370 inhibitor suppressed mature miR-370 expression to $31\% \pm 0.04$ ($p < 0.05$) and $58\% \pm 0.05$ ($p < 0.05$) lower in HL60 and K562 cells, respectively (Figure 2B). The decline in miR-370 expression was coupled with enhanced cell proliferation (Figure 2C) (pSuper vs pSuper-miR-inhibitor: HL60: 56 ± 7 vs 72 ± 6, $p < 0.05$; K562: 66 ± 12 vs 93 ± 7, $p < 0.05$).

The above result suggests that miR-370 suppresses proliferation of HL60 and K562 cells. We further wanted to define the mechanism behind miR-370 overexpression-mediated proliferation inhibition. We suspected that miR-370 might trigger cellular senescence program. Senescence-associated β-Gal staining, a specific marker for senescent cells [19], was thus performed. A positive
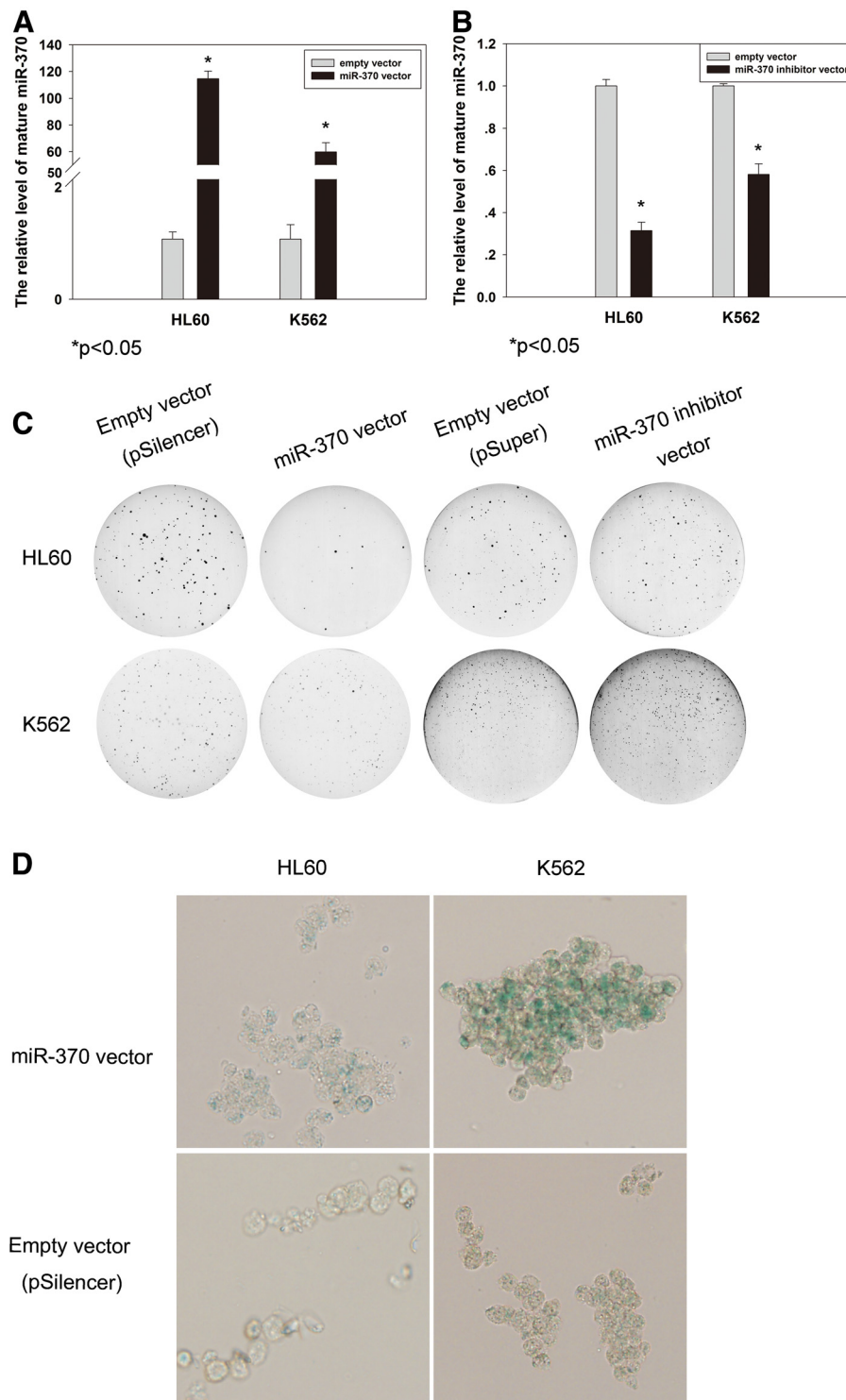

Figure 1 Downexpression of miR-370 in de novo AML patients. (**A**) *miR-370* expression by qRT-PCR in 48 de novo AML patients, 40 AML patients of 1st CR and 21 healthy controls. (**B**) Restoration of miR-370 expression in six patients after complete remission achievement in induction chemotherapy. The results are shown as miRNA expression after normalization with U6 and -ΔCt calculations.

Figure 2 (See legend on next page.)

β-Gal staining was observed in the two cell lines transfected with miR-370 precursors (Figure 2D) [pSilencer vs pSilencer-miR (% of β-Gal-positive cells): HL60: 3 ± 1 vs 28 ± 3, p < 0.01; K562: 8 ± 3 vs 40 ± 1, p < 0.01;].

Up-regulation of miR-370 expression mediated by 5-Aza-CdR

DNA methylation is an epigenetic modification that regulates gene expression. Aberrant DNA methylation has

(See figure on previous page.)
**Figure 2 Suppression of AML cell proliferation and induction of cell senescence by overexpression of miR-370 in vitro while
anti-miR-370 expression increases cellular ability of proliferation.** (**A**) *miR-370* expression in AML cell lines and its overexpression using a
specific miR-expressing pSilencer vector. The cells were transfected with either miR-370 precursors or with control precursor and harvested after
48 hours. qRT-PCR was applied to detect mature *miR-370* level. (**B**) The depletion of *miR-370* expression by a specific *miR-370*-inhibiting pSuper
plasmid. The method was similar as above. (**C**) Alterations in foci formation after different treatments in AML cells. The cells were first treated as
above and the efficiently changed *miR-370* expression was verified in (A) and (B). For clonogenic assays, 5,000 cells/well (in 6-well plates) were
incubated for 14 days and the foci number was counted. (**D**) Senescence onset of *miR-370*-overexpressed AML cells. β-Gal staining was
performed and % of positive cells was calculated.

been implicated in many cancers [20]. Global hypo-
methylation or aberrant hypermethylation of gene pro-
moter CpG islands result, respectively, in tumor cell
genomic instability and gene silencing, particularly of
tumor suppressor genes [17]. Interestingly, the chromo-
somal location of miR-370 on chromosome 14q32.31
has been shown to be regulated by DNA methylation, or
deleted by loss of heterozygosity [14,21] or by hyper-
methylation of an CpG island 200 bp upstream in the
mother allele [22]. Treatment with 5 μM 5-aza-CdR, a
DNA methylation inhibitor, for 72 hours, substantially
(>2.0-fold) and significantly (P < 0.05) increased the ex-
pression of miR-370 in both HL60 and K562 cells
(Figure 3A) and decreased cell proliferation (Figure 3D)
(control vs CdR: HL60: 24 ± 4 vs 7 ± 2, p < 0.01; K562:
152 ± 5 vs 78 ± 5, p < 0.001).

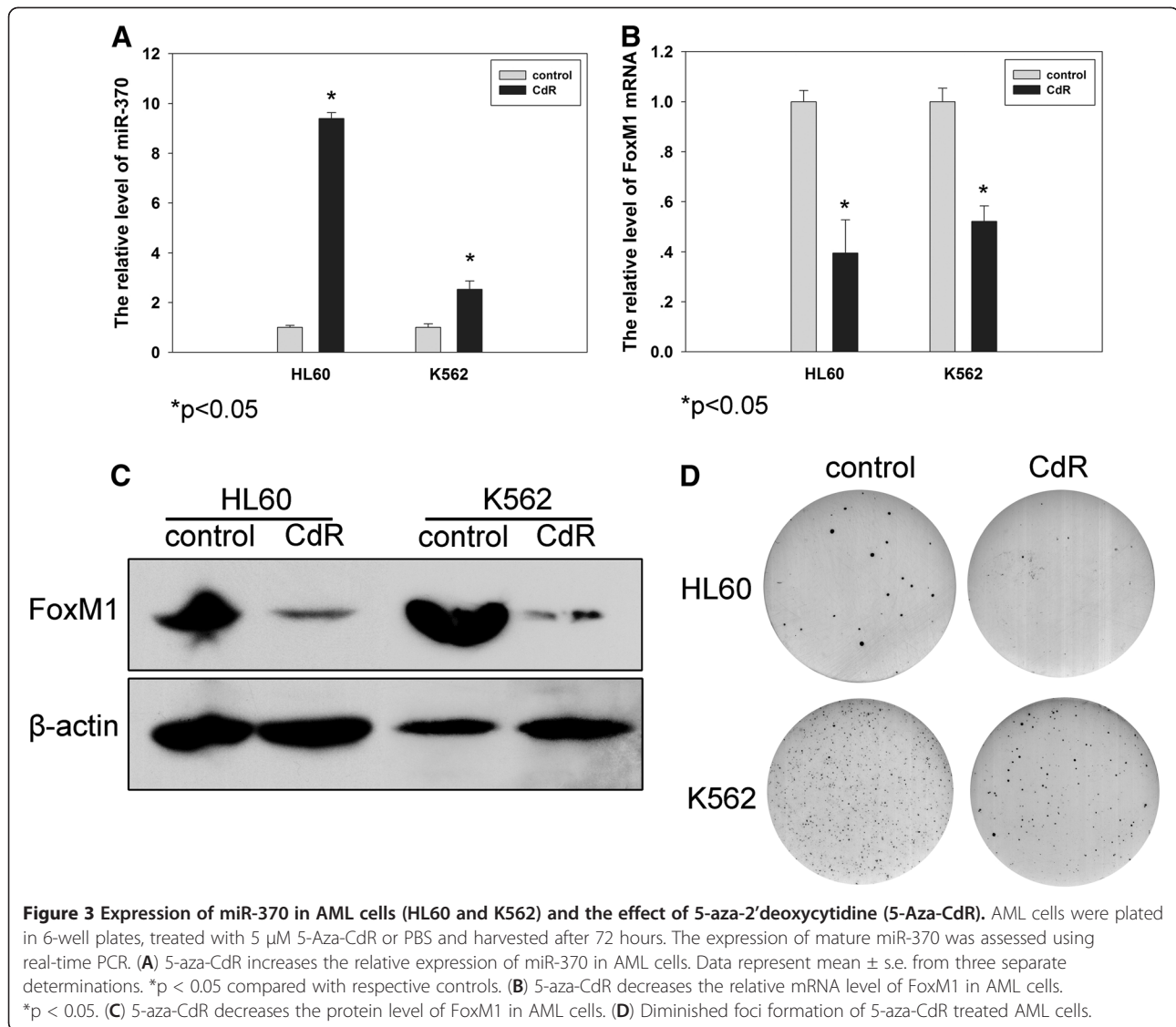### Identification of FoxM1 as a target for miR-370
To further elucidate the mechanism by which miR-370
affected cellular senescence and proliferation, we next
screened for potential targets of miR-370 using four tar-
get prediction programs with different algorithms:
DIANA-MicroT [23], TargetScan [24], Miranda [25] and
PicTar [26]. All potential targets predicted by more than
one of these programs were identified. We selected the
forkhead box M1 (FoxM1) for further study because of
its well-characterized role in tumor biology. The FoxM1
gene has a 249-bp 3′UTR region that presents a 7-mer
binding site for miR-370 (Figure 4A).

First, we made the luciferase reporter constructs con-
taining the miR-370 recognition sequence from the 3′-
UTR of FoxM1 inserted downstream of the luciferase
gene. Transfection with miR-370 precursor decreased re-
porter activity in K562 cells (Figure 4B), which strongly
indicates that FoxM1 is a target for miR-370. Next, the
studies were repeated with random mutations in the rec-
ognition sequence (Figure 4A), which resulted in aboli-
tion of the reporter activation by miR-370 precursor
(Figure 4B). Finally, we assessed the effect of miR-370
expression on FoxM1 expression. Transfection of HL60
and K562 cells with miR-370 precursor resulted in lower
expression of FoxM1 after 48 hours (Figure 4C-D). Con-
comitant with decreased FoxM1 expression, there was
reduction of its downstream target *c-myc* and *skp2*

(Figure 4C-D). There was a >2-fold increase in expres-
sion of FoxM1 in HL60 and K562 cells after transfection
of miR370 inhibitor plasimids (Figure 4E). 5-aza-CdR
significantly reduced the expression of FoxM1 in both
HL60 and K562 cells (Figure 3 B-C). These changes
were similar to those observed with miR-370 overex-
pression. Taken together, FoxM1 is a target of miR-370.

Overexpression of FoxM1 in de novo AML patients
FoxM1, a master regulator of mitotic gene expression, is
required for cell proliferation and its inhibition leads to
reduction in anchor-independent growth and tumori-
genesis of cancer cells [27]. As we have verified that
FoxM1 is a target for miR-370, we then sought to probe
its role in AML. The tumor specimens from forty-eight
de novo AML patients and forty AML patients in 1st
CR and twenty-one healthy controls were analyzed for
FoxM1 mRNA expression using qRT-PCR. Patient char-
acteristics are described in Table 1. The FoxM1 tran-
script level in AML patients was found 21.47-fold higher
than that in controls, while following acquisition of CR
in the induction chemotherapy, FoxM1 expression level
reduced to 1.75 fold of controls. (Figure 5 A, C), which
was negatively correlated with miR-370 levels. There was
a highly significant difference in FoxM1 expression be-
tween AML samples, CR samples and healthy controls
(qRT-PCR, One-Way ANOVA, p < 0.01). In six patients,
BM samples were available both at diagnosis time prior
to treatment and after a complete remission and we
found a higher FoxM1 level at diagnosis while a signifi-
cant decrease in FoxM1 expression after CR except one
sample (Figure 5B). There was no clear association be-
tween the presence of FoxM1 mRNA and age, gender,
tumor burden or FAB subtypes (data not shown). More-
over, BM materials from 8 de novo AML patients, 8
patients in 1st CR and 5 healthy controls randomly
chosen from our AML patient pool were used to deter-
mine the mRNA expression level of *c-Myc*, *hTERT*,
p27^{kip1} and *skp2*, all of which were the target genes of
FoxM1, using real-time PCR method. We found that the
transcript levels of *c-Myc*, *hTERT* and *skp2* in AML
patients were respectively 9.64, 3.76 and 3.14-fold higher
than those in controls, while following acquisition of
CR, all of them reduced almost to the same levels of

Figure 3 Expression of miR-370 in AML cells (HL60 and K562) and the effect of 5-aza-2'deoxycytidine (5-Aza-CdR). AML cells were plated in 6-well plates, treated with 5 μM 5-Aza-CdR or PBS and harvested after 72 hours. The expression of mature miR-370 was assessed using real-time PCR. (**A**) 5-aza-CdR increases the relative expression of miR-370 in AML cells. Data represent mean ± s.e. from three separate determinations. *p < 0.05 compared with respective controls. (**B**) 5-aza-CdR decreases the relative mRNA level of FoxM1 in AML cells. *p < 0.05. (**C**) 5-aza-CdR decreases the protein level of FoxM1 in AML cells. (**D**) Diminished foci formation of 5-aza-CdR treated AML cells.

controls. On the contrary, the expression level of $p27^{kip1}$ in AML patients is only 40% of that in controls, while restored after CR (Additional file 2).

Defective foci formation by depletion of FoxM1 in AML cell lines
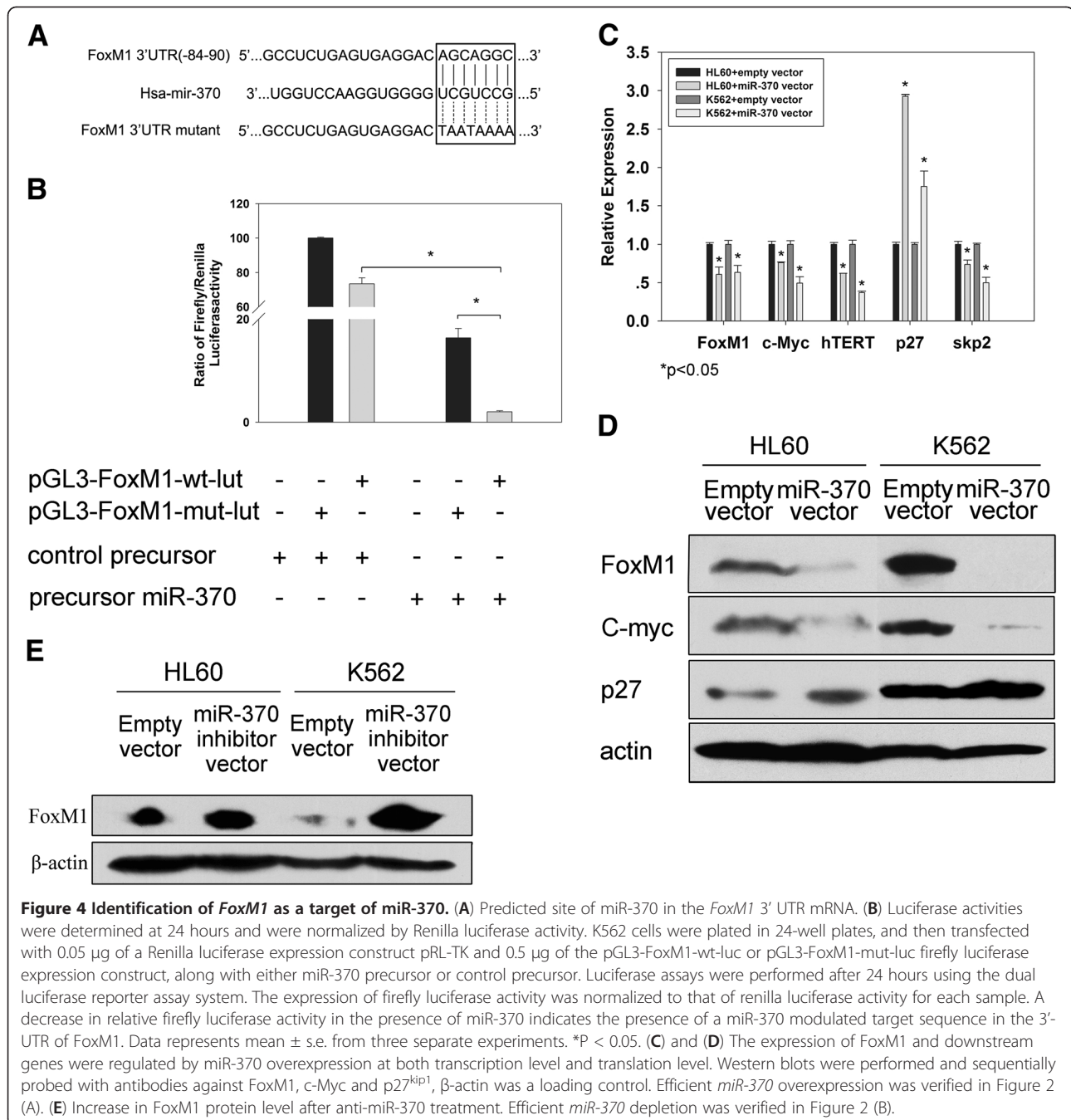
Then, we employed the FoxM1-specific siRNA to knock down FoxM1 expression in AML cell lines and the cells were then assessed for their clonogenic capacity. Efficient silence of FoxM1 expression in these cells was verified using qRT-PCR and Western blot analyses (Figure 5 D-E). Compared to the control cells, the FoxM1-knocked down cells exhibited significantly diminished foci formation (Figure 5F) (Controls vs FoxM1 siRNA: HL60: 19 ± 3 vs 11 ± 2, p < 0.05; K562: 33 ± 5 vs 5 ± 2, p = 0.001). Following FoxM1 depletion, its target genes *c-myc, skp2* and *hTERT* were also down-

regulated and coupled with the accumulation of the CDK inhibitor $p27^{kip1}$ (Figure 5 D-E).

Collectively, the *FoxM1* gene is aberrantly activated in AML and is required for sustained proliferation of the cancer cells.

Discussion

In the present study, we explored the expression and role of miR-370 in AML. Our findings show a down-regulation of miR-370 in blasts from patients with de novo AML. Moreover, we identified FoxM1 as a target for miR-370 and restored expression of miR-370 reduced the level of FoxM1. In hematological malignancies a link with miRNA was initially described in chronic lympho-cytic leukemia (CLL) by Calin et al. [28]. A cluster of two miRNAs, miR-15a, and miR-16, was found to be located within the deleted region at 13q14, and down-

Figure 4 Identification of *FoxM1* as a target of miR-370. (**A**) Predicted site of miR-370 in the *FoxM1* 3' UTR mRNA. (**B**) Luciferase activities were determined at 24 hours and were normalized by Renilla luciferase activity. K562 cells were plated in 24-well plates, and then transfected with 0.05 μg of a Renilla luciferase expression construct pRL-TK and 0.5 μg of the pGL3-FoxM1-wt-luc or pGL3-FoxM1-mut-luc firefly luciferase expression construct, along with either miR-370 precursor or control precursor. Luciferase assays were performed after 24 hours using the dual luciferase reporter assay system. The expression of firefly luciferase activity was normalized to that of renilla luciferase activity for each sample. A decrease in relative firefly luciferase activity in the presence of miR-370 indicates the presence of a miR-370 modulated target sequence in the 3'-UTR of FoxM1. Data represents mean ± s.e. from three separate experiments. *P < 0.05. (**C**) and (**D**) The expression of FoxM1 and downstream genes were regulated by miR-370 overexpression at both transcription level and translation level. Western blots were performed and sequentially probed with antibodies against FoxM1, c-Myc and p27[kip1], β-actin was a loading control. Efficient *miR-370* overexpression was verified in Figure 2 (A). (**E**) Increase in FoxM1 protein level after anti-miR-370 treatment. Efficient *miR-370* depletion was verified in Figure 2 (B).

regulated in the majority (70%) of CLL samples. Notably, miR-15a and miR-16-1 down-regulation contribute to malignant transformation by up-regulating BCL2 [29]. Recently, involvement of miRNA in AML has been documented identifying that miRNA expression profiles are AML subtype-specific and suggesting a pathogenetic role of miRNA in AML. For example, Mi and colleagues [30] showed distinct miRNA signatures between acute lymphoblastic leukemias (ALLs) and AMLs. In that study, patients with AML could be separated from those with ALL on the basis of 21 up-regulated and 6 down-regulated miRNAs, among which four of them (let-7b, miR-128a, miR-128b and miR-223) were the most discriminatory [31]. According to Amanda Dixon-McIver, compared with other major cytogenetic subgroups of AML, APLs bearing the t(15;17) translocation were characterised by the up regulation of 7 miRNAs transcribed from genes located at the 14q32 region. The set includes miR-127, miR-154, miR-154*, miR-299, miR-323, miR-368, and miR-370. Two other groups reported

Table 1 Patient Characteristics

Characteristic	De novo AML patients	AML patients in 1st CR
Patients,no.	48	40
Sex,male/female,no.(%)	31(65)/17(35)	24(60)/16(40)
Median age,y(range)	41(14–74)	41(14–68)
WBC count (10^9/L)		
Median	46.3	6.3
Range	0.8-354.9	2.6-10.2
Hemoglobin (g/dL)		
Median	7.7	11.8
Range	4.1-13.2	8.3-16.1
Platelet count (10^9/L)		
Median	58	243.3
Range	2-319	76-384
BM blasts (%)		
Median	69.1	2.1
Range	(24–96)	(0–5)
FAB subtype,n/(%)		
M2	9/(19)	6/(15)
M3	11/(23)	20/(50)
M4	11/(23)	8/(20)
M5	15/(31)	5/(12.5)
M6	2/(4)	1/(2.5)

independently that miR-155 was up-regulated in AML patients with FLT3-ITD, suggesting that this miRNA contributes to the highly proliferative phenotype of this molecular subset of AML [31-33]. In the present study, we haven't got the conclusion that the expression level of miR-370 is with AML subtype-specificity, which may be due to the limited number of primary AML patients enrolled in our study.

DNA methylation is an epigenetic modification that can regulate gene expression. Aberrant DNA methylation has been implicated in many cancers [21]. miR-370 and many other miRNAs are organised in clusters together on chromosome 14q32 [34]. This miRNA cluster acts as imprinted non-coding RNA (ncRNA) genes, which are mono-allelically expressed in a parent-of-origin manner (their expression is restricted to either the maternal or the paternal allele). Interestingly, as for this miRNA cluster, which is of maternal origin, its imprinted expression is regulated by an intergenic differentially methylated region (DMR) located ~ 200 kb upstream from the miRNA cluster [24]. Hypermethylation of DMR causes silence of this miRNA cluster, including miR-370. It has been suggested that miRNAs in this region act as tumour repressor genes and that changes in the methylation status of their promoters could trigger
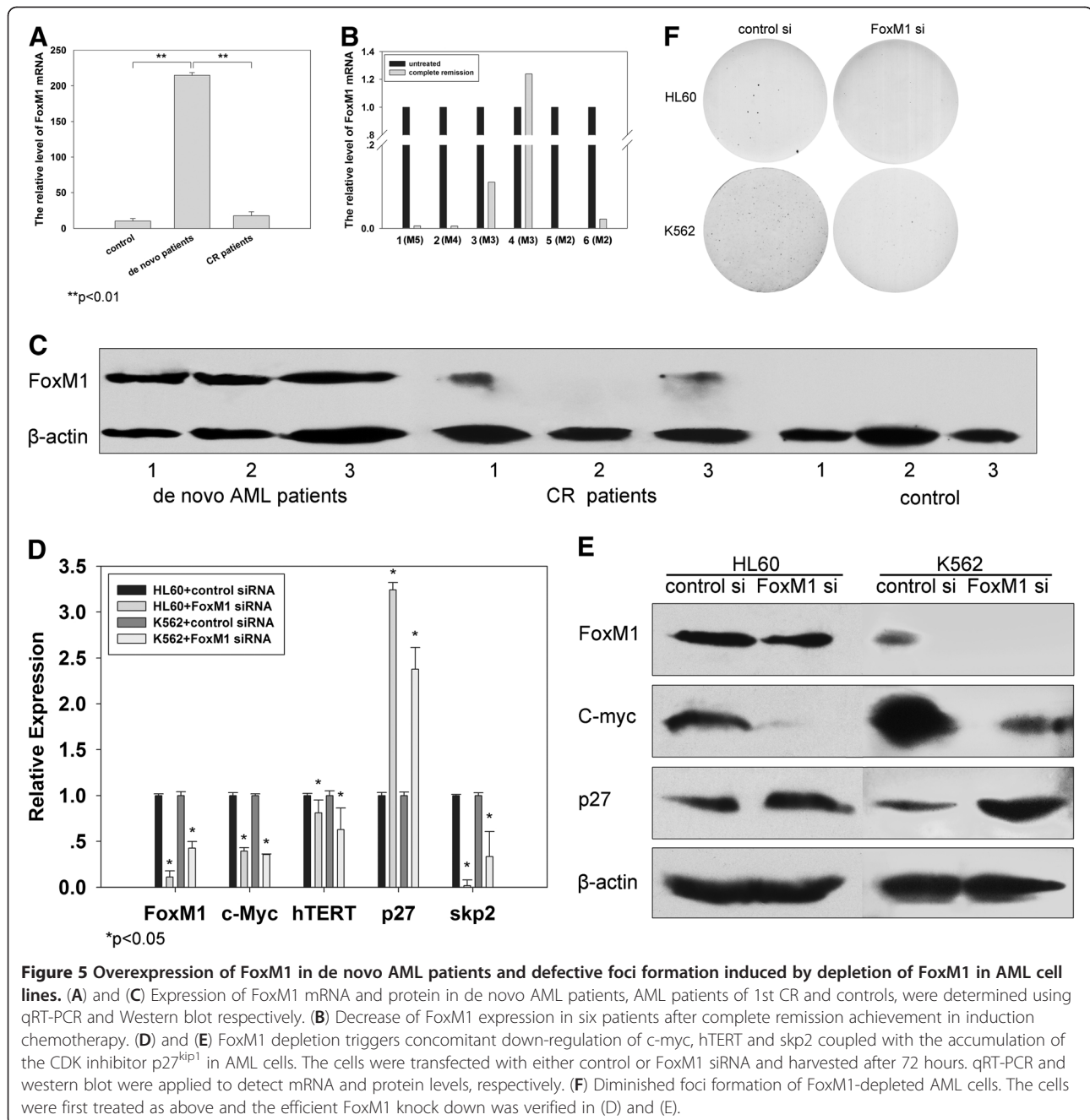
cancer development [35]. For instance, miR-127 has been shown to be down regulated or silenced in cancer cells, whose expression is correlated with the methylation and acetylation status of its promoter. Inhibition of methylation and histone deacetylation in these cancer cells causes over expression of miR-127 and related down regulation of the target BCL6, a bona fide protooncogene [36]. We hypothesize that miR-370 also acts as a tumor suppressor in AML, as in papillary thyroid carcinoma, colorectal cancer and malignant cholangiocytes. The comparison of the leukemia samples with healthy controls highlighted the differential expression of miR-370. Following the treatment with 5-aza-2′-deoxycytidine, there is a significant enrichment for miR-370 in AML cell lines, which indicated that hypermethylation may contribute to reduction of miR-370.

Cancer therapy has traditionally relied on cytotoxic treatment strategies on the assumption that complete cellular destruction of tumors optimizes the potential for patient survival. Although these approaches produce complete cell death within a tumor, they also can cause severe side effects in patients [37]. Recently, a promising approach to preventing continued tumor growth is therapy-induced senescence (TIS) [38]. Senescent cells remain viable and metabolically active but are permanently growth arrested [39]. Evidence has recently accumulated that cellular senescence is a potent barrier to cancer development. Our results indeed demonstrated that senescence occurred in most of AML cells treated with miR-370 overexpressing plasmid, which was concomitant with their diminished clonogenic capacity. Given a critical role for senescence induction in tumor suppression and therapeutic efficacy of cancer treatment, the present findings have important biological and clinical implications. All these results suggest that downregulation of miR-370 may be another mechanism involved in the pathology of AML and therefore, could be used as a diagnostic marker and therapeutic target in AML.

We have also analyzed the correlation between miR-370 expression and FoxM1 mRNA expression in 48 de novo AML samples. Consistent with the cell line data, FoxM1 was enriched in the primary blasts mRNAs that inversely correlated to miR-370 expression levels. This result was also observed at the protein level in a few primary AML samples. However, further studies using large numbers of primary AML samples will be needed to confirm this interaction.

Conclusion

We demonstrate that miR-370 is a tumor suppressive factor by targeting multiple critical oncogenic pathways. Restoring miR-370 expression downmodulates FoxM1,

Figure 5 Overexpression of FoxM1 in de novo AML patients and defective foci formation induced by depletion of FoxM1 in AML cell lines. (**A**) and (**C**) Expression of FoxM1 mRNA and protein in de novo AML patients, AML patients of 1st CR and controls, were determined using qRT-PCR and Western blot respectively. (**B**) Decrease of FoxM1 expression in six patients after complete remission achievement in induction chemotherapy. (**D**) and (**E**) FoxM1 depletion triggers concomitant down-regulation of c-myc, hTERT and skp2 coupled with the accumulation of the CDK inhibitor p27^{kip1} in AML cells. The cells were transfected with either control or FoxM1 siRNA and harvested after 72 hours. qRT-PCR and western blot were applied to detect mRNA and protein levels, respectively. (**F**) Diminished foci formation of FoxM1-depleted AML cells. The cells were first treated as above and the efficient FoxM1 knock down was verified in (D) and (E).

induces senescence, and dampens cell growth in AML cells, thereby suggesting miRNA-based therapy as a novel approach to increase response in AML.

Materials and methods
Patients and bone marrow samples
Forty-eight newly diagnosed AML patients (31 male and 17 female; median age at diagnosis 41; range 14–74), forty AML patients in 1st complete remission (CR) and twenty-one healthy controls were enrolled in this study. Diagnosis of AML was established according to clinical

presentation and morphologic criteria of the French-American-British (FAB) Classification. The study was approved by the local ethics committee. Patients' BM samples were collected between April 2008 and September 2011 at the Department of Hematology, Qilu Hospital, Shandong University, Jinan, China. Mononuclear cells were isolated using Ficoll-Hypaque density gradient centrifugation, and then stored at –80°C until use. All patients and healthy controls were tested for miR-370 and FoxM1 mRNA levels in their BM cells. Among those AML patients, six were analyzed for miR-370 and

FoxM1 levels in their bone marrow samples at both diagnosis and complete remission.

Cell lines and culture conditions

Human AML cell lines HL60 and K562 were cultured at 37°C, 95% air, 5% CO_2 in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 100 μg/mL penicillin, and 50 μg/mL streptomycin. To assess 5-aza-CdR (Sigma, Santa Clara, CA, USA) effects, cells were grown on 6-well plates, treated with 5 μM 5-aza-CdR or cold phosphate-buffered saline (PBS) controls for 72 h at 37°C, and then harvested for isolation of total mRNA or protein.

RNA extraction and quantitative real-time PCR

Total cellular RNA in BM samples and in cells with different treatments was extracted using the Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using random primers (N6) (Fermentas, St. Leon-Rot, Germany) and MMLV reverse transcriptase. The PCR primers used in the study were as follows: sequences specific for FoxM1 mRNA: 5′-TGCAGCTAGGATGTGAATCTTC-3′ (Forward) and 5′-GGAGCCCAGTCCATCAGAACT-3′ (Reverse). Skp2: 5′-GGACCTATCGAACTCAGTTAT-3′ (Forward) and 5′-CAGCCACCTGTACATGCTTT-3′ (Reverse); p27^{kip}: 5′-ATGTCAAACGTGCGAGTGTCTAA-3′ (Forward) and 5′-TTACGTTTGACGTCTTCTGAGG-3′ (Reverse); hTERT: 5′-CGGAAGAGTGTCTGGAGCAA-3′ (Forward) and 5′-GGATGAAGCGGAGTCTGGA-3′ (Reverse); c-MYC: 5′-TACCCTCTCAACGACAGCAGCTCGCCCAACTCCT-3′ (Forward) and 5′-TCTTGACATTCTCCTCGGTGTCCGAGGACCT-3′ (Reverse). The above primer pairs cross intron/exon boundaries; thus, the resultant PCR products do not represent genomic DNA contamination. β-actin expression was used as a control for RNA loading and RT efficiency and amplified. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in an ABI7000 sequence detector (Applied Biosystems, Foster City, CA, USA).

TaqMan qRT-PCR miRNA analysis

Quantification of mature miRNAs was performed using qRT-PCR with the TaqMan miRNA assay kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instruction. Briefly, 10 ng of total RNA was reverse-transcribed (RT) with specific primers, subsequently 1.5 μL of RT product was used as template for real-time PCR. All real-time experiments were performed in triplicate. Data were normalized by the expression of small nuclear RNA (snRNA) U6 and expressed either as relative expression ($2^{-\Delta Ct}$) or as fold change relative to control ($2^{-\Delta\Delta Ct}$).

Western blot

Total cellular proteins were extracted from cultured cells or BM samples. Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were probed with the specific antibodies against FoxM1, p27, c-MYC (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), followed by anti-mouse or rabbit horseradish peroxidase–conjugated IgG and developed with the enhanced chemiluminescence method (ECL). β-actin served as a loading control.

miR-expressing and miR-inhibiting plasmids

To generate the miR-expressing pSilencer3.1-H1 neo vector, a fragment of 212 base pairs (bp) corresponding to the desired miRNA and the surrounding sequences was amplified from human genomic sequence, adding a BamHI site and a HindIII site to the 5′ and 3′ ends respectively, using the polymerase chain reaction (PCR) with primers sense: 5′ AAGGGATCCTACTTGAGG GATGGGCGATA 3′ and antisense 5′ TCAAAGCTTC CCGAGCTCTGGTGTTAGAC 3′. We included a large portion of miRNA surrounding sequence in the attempt to allow correct processing of the miRNA to its mature form and to induce overexpression while preserving a physiologic mechanism of miRNA production. miR-370 inhibitor sequences were synthesized as DNA oligonucleotides; after annealing, were sticking ended and subcloned into a pSuper vector.

Transfection

Cells were incubated in 6-well plates (3.0×10^5/well and 1.0×10^5/well, respectively) overnight and were then transfected with plasmid or siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Chemical modified Stealth small interfering RNA (siRNA) targeting FoxM1 and control siRNA were bought from Invitrogen. The sequence for the FoxM1 siRNA was 5′-GACAACUGUCAAGUGUAC CACUCUU-3′.

Soft agar colony formation assay

HL60 and K562 cells were resuspended in DMEM (Gibco, Carlsbad, CA, USA) containing 20% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) with equal amount of either 0.3% agar (HL60 cells) or 0.5% agar (K562 cells), and plated in 6-well plates at 5,000 per well on top of a 2 mL precast semisolid 1% agar underlayer as described previously [40]. The number of colonies with more than 50 cells was counted after two weeks.

Senescence-associated β-Galactosidase (SA -β-Gal) staining

SA -β-Gal staining was done as described [19,41]. Briefly, the cells grown in 6-well plates were transfected with

pSilencer or pSilencer-miR-370. After 7 days, the cells were rinsed with PBS once, fixed in 3% of formaldehyde for 15 min, and incubated with freshly prepared SA -β-Gal staining solution at 37°C overnight.

Luciferase reporter vector

The precursor to miR-370 was synthesized and cloned in pSilencer. Firefly luciferase reporter vectors with the intact putative miR-370 recognition sequence from the 3′-UTR of FoxM1 (pGL3-FoxM1-wt-3′-UTR) or with random mutations (pGL3-FoxM1-mut-3′-UTR) cloned downstream of the firefly luciferase gene were constructed. Wild-type and mutant inserts were confirmed by sequencing. For the 3′UTR-luciferase assays, cells were co-transfected with 0.5 μg pGL3-FoxM1-wt or mut-3′-UTR construct, 4 μg of pSilencer or pSilencer-miR and 0.05 μg pRL-TK Renilla luciferase expression construct using Lipofectamine 2000. Luciferase assays were performed 24 h after transfection using the Dual Luciferase Reporter Assay system (Promega, Madison, Wisconsin, USA).

Statistical analyses

The difference in miR-370 and FoxM1 mRNA expression among different patient groups as detected using qRT-PCR was analyzed using One-Way ANOVA. The comparison of foci numbers, β-Gal-positive cells, luciferase activity and miR-370, FoxM1, c-myc, hTERT, p27, skp2 mRNA expression after different treatments was made using a Student's t-test. All the tests were two-tailed and computed using SPSS11.5 software. Results are depicted as the mean ± standard error of the mean. P values < 0.05 were defined as statistical significance.

Additional files

Additional file 1: Proliferation curve of HL60 cell line (A) and K562 cell line (B) after transfection with miR-370-expressing plasmid or the control pSilencer vector. 1×10^5/ml cells were plated in 6-well plates just before the transfection. The number of viable cells was counted at 24 h and 48 h points using trypan blue. Data represents mean ± s.e. from three separate experiments. **p < 0.01 Student's t-test.

Additional file 2: c-Myc, hTERT, p27^{kip1} and skp2 expression by qRT-PCR in 8 de novo AML patients, 8 AML patients of 1st CR and 5 healthy controls. The transcript levels of c-Myc, hTERT and skp2 in AML patients were found respectively 9.64, 3.76 and 3.14-fold higher than those in controls, while following acquisition of CR in the induction chemotherapy, all of them reduced almost to the same levels of controls. On the contrary, the expression level of p27^{kip1} in AML patients is only 40% of that in controls, while restored after CR. *p < 0.05 One-Way ANOVA.

Competing interests

The authors declare no competing financial interests.

Authors' contributions

JZ, CC and XZ designed the study; XZ, JZ, BL, MZ, YZ and TH performed the research; XZ ,JZ, LW, JJ, and CC analyzed and interpreted data; and XZ, JZ and CC wrote the paper. All authors read and approved the final manuscript.

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