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Original Article

Knockdown of SOX12 Expression Induced Apoptotic Factors is Associated with TWIST1 and CTNNB1 Expression in Human Acute Myeloid Leukemia Cells

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Recent improvements in molecular treatment and gene therapy led to discovering novel cancer remedies. Antisense LNA GapmeRs is a state-of-the-art molecular research field for diagnosing and treating various cancer types. Acute myeloid leukemia (AML) is a heterogeneous hematopoietic malignancy defined by the rapid accumulation and malignant proliferation of immature myeloid progenitors. *SOX12* is a new potential target for acute myeloid leukemia. In this study, *SOX12* was blocked by antisense LNA GapmeRs (ALG) in human AML cell lines (KG1 and M07e). Cells were transfected with Gapmer anti-*SOX12* at 24, 48, and 72 h post-transfection. Transfection efficiency was assessed by a fluorescent microscope. Furthermore, evaluation of *SOX12*, *TWIST1*, *CTNNB1*, *CASP3*, and *CASP9* expression was performed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Cell viability was determined by MTT assay. *SOX12* expression was decreased remarkably in the ALG group. Moreover, *SOX12* knockdown was associated with a decrease in *TWIST1* and *CTNNB1* expression. Besides, downregulation of *SOX12* in both cell lines could induce apoptosis, probably through upregulation of *CASP3* and *CASP9*. The findings reveal that *SOX12* knockdown could be a new target for reducing AML cells proliferation through antisense therapy approach.

Key words: Acute myeloblastic leukemia, antisense LNA GapmeRs, *SOX12*, *TWIST1*, *CTNNB1*, apoptosis

Leukemias are a group of progressive, malignant hematological diseases that consist of abnormally differentiated oligoclonal expansions and sometimes poorly differentiated hematopoietic cells that could invade the blood and other extramedullary tissues (1). The highest percentage of patients with acute myeloid leukemia (AML) belong to adults (2). Although induction chemotherapy is an effective treatment, relapse is one of the severe causes of treatment failure (3). Based on the French–American–British (FAB) classification, AML has nine subtypes (M0, M1,

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M2, M3, M4, M4E0, M5, M6, and M7) according to cytochemical staining, morphological characteristics of the cancerous cells (2). Mutations in different genes such as nucleophosmin 1 (*NPM1*), CCAAT enhancer binding protein alpha (*CEBPA*), runt-related transcription factor 1 (*RUNX1*), *KIT* receptor tyrosine kinase, and fms-like tyrosine kinase 3 (*FLT3*) are sometimes a cause of the incidence of AML (4).

Dysfunction of some specific transcription factors due to mutations is also an important cause of AML. New treatments for AML that target the appropriate transcription machinery are potentially effective (5, 6). Since the functions and activities of most transcription factors in AML remain elusive, the identification of these factors could give visions into the diagnosis and treatment of AML. The SRY-related high mobility group (HMG) box (*SOX*) gene family encodes a group of transcription factors having critical roles in the primary cellular processes during development (7). Structural abnormalities in *SOX* genes have been correlated with different types of cancer (8). *SOX* genes are divided into A-H sub-groups based on the HMG box. SOXC group consists of *SOX4*, *SOX11*, and *SOX12*. Among them, *SOX4* has a pivotal role in regulating the normal function of hematopoietic stem cells, and its overexpression is a joint event in AML malignancy (9). *SOX11* expression is illustrated in lymphoblastic lymphoma, some Burkitt lymphomas, and T-prolymphocytic leukemia (10). *SOX12* (previously named *SOX22*) is expressed in several fetal and adult tissues and organs, and has an indispensable role in the differentiation and maintenance of different cell types. It is a direct target for forkhead box Q1 (FOXQ1) that promotes metastasis (11). The role of the canonical WNT/β-catenin signaling pathway is essential for regulating tissue development and maintaining homeostasis. As a result, the dysfunction of this pathway can cause different diseases, including cancer. β-catenin (CTNNB1) is a critical transcriptional co-activator in the canonical WNT signaling pathway and can firmly regulate cell-to-cell adhesion (12). The *SOX* genes' functions are mostly associated with the Wnt/β-catenin (WNT/CTNNB1) pathway by regulating β-catenin expression or its binding to *WNT* gene promoters as a target to control protein stability and nuclear translocation. Dysfunction in this pathway has a defined role in the AML incidence (13). Twist family bHLH transcription factor 1 (TWIST1) is a crucial factor that induces epithelial-mesenchymal transition (EMT). Several experiments showed that TWIST1 raises tumor metastasis and demonstrated poor prognosis in various human cancers (14). On the other hand, studies reported that the expression of several metastatic-related genes such as *TWIST1* increased by up-regulation of *SOX12* (15).

Antisense LNA GapmeRs are a new generation of DNA antisense oligonucleotides that contain locked nucleic acid (LNA) and induce the degradation of target sequence via RNase H-dependent mechanism (16). To date, not many studies have been reported on the potential function of *SOX12* in AML. In this study, we investigated the potential role of *SOX12* in AML by knocking down its expression in two AML cell lines by using antisense LNA GapmeR, and by assessing the expression of related genes, *TWIST1* and *CTNNB1*, in the cell populations. Also, expressions of CASP3 and CASP9 as apoptotic factors were investigated.

Materials and methods

Cell culture

KG1 cell line (human acute myeloid leukemia, AML M6) and M07e cell line (human megakaryocytic cell line) were purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Complete medium including RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 10% v/v fetal bovine serum (FBS; Gibco, UK) and 1% penicillin/streptomycin (Sigma-Aldrich, Saint Louis, MO, USA), were used to culture

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KG1cells. Additionally, for M07e cells, this media was complemented with five ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN, USA). Cells were cultured in a 25-cm^2 cell culture flask (Nunc, Roskilde, Denmark) and incubated at 37 °C with a 5% CO_2 atmosphere. The cells were passaged once a week to maintain the exponential phase.

The local ethics committee approved this study of Isfahan University of Medical Sciences (IRAN), and the study has been approved by the appropriate institutional and national research ethics committee. It has been performed by the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Cell transfection

The LNA targeting sequence of the *SOX12* gene was acquired from a reputable site: http://www.Incrnadb.org as taCTTGTAGTCCGG GTAAtc. Antisense LNA GapmeRs (ALG) and antisense LNA GapmeR negative control oligonucleotides (ALGNC) for *SOX12* were purchased from Eurofins. ALG and ALGNC were labeled at their 5'end with 6-FAM (6-carboxyfluorescein), which is a fluorescent dye. KG1 and M07e cells were transfected by using the PolyFect™ transfection reagent kit (Qiagen, Germany) pursuant to the manufacturer's guidance. In summary, a total number of about 5 × 10^5 cells of each line, in the exponential phase, were seeded into six-well culture plates (Nunc, Denmark) containing 1.8 mL RPMI 1640 per well without antibiotics and FBS. Two different microtubes were labeled as G (GapmeR) and T (Transfect). Each cell well contained 100 µL RPMI. 6 µL LNA GapmeRper from G microtube and 3 µL of transfection reagent from T microtube were added to each cell well. After pipetting well, the transfection reagent was added into antisense LNA GapmeR and incubated at room temperature in the dark for 15 min, then 200 µL of this reagent was added to each cell well while the plates were rotated to ensure that all the reagent and cell culture media were mixed thoroughly. We seeded 3 wells for each cell line labeled as GapmeR, Negative, and control (without LNA GapmeR and ALGNC, containing only the cells). After 6 h incubation, 100 µL FBS and 10 µL antibiotics were added to each well. Finally, the cells were incubated for 24, 48, and 72 h. Transfected cells were measured by fluorescence microscopy and flow cytometry due to the fluorescent dye (6-FAM) (17).

Real-time PCR

Real-time PCR was used to assess the efficiency of the *SOX12* inhibition by ALG. Briefly, total RNA of both KG1 and M07e cells were extracted with RNA extraction kit (Biofact, Korea) at 24, 48, and 72 h after transfection. RNA concentration and purity were determined on a spectrophotometer by calculating the optical density ratio (OD) at 260 and 280 nm wavelengths. Complementary DNA (cDNA) was then synthesized using the universal cDNA Synthesis Kit (Biofact, Korea). The SYBR green master mix kit was used for real-time PCR analysis (Biofact, Korea). The reference gene for normalization of the expression level was *GAPDH*, and all assays were carried out in triplicate. The forward and reverse primers for the selected genes were listed in Table 1. The annealing temperature was 60 ºC for all studied genes and 40 amplification cycles were performed for each real-time PCR experiment in the ABI Step One Plus (ABI, USA) instrument, and the relative expression levels were analyzed by the ΔΔCt method.

Table 1. Sequences of primer pairs used for real-time RT-PCR method.

Primer name	Sequences (5′–3′)
***SOX12*-F**	CTGGAGTGGTGGGATTGGTC
***SOX12*-R**	GGGTGTCAGAGGGACAAAGG
***TWIST1*-F**	TCTCGGTCTGGAGGATGGAG
***TWIST1*-R**	AATGACATCTAGGTCTCCGGC
***CTNNB1*-F**	CAACCAAGAAAGCAAGCTCATC
***CTNNB1*-R**	CAGATAGCACCTTCAGCACTC
***CASP3*-F**	TCCACAGCACCTGGTTATTATTC
***CASP3*-R**	ACTCAAATTCTGTTGCCACCTTTC
***CASP9*-F**	ATTTGGTGATGTCGGTGCTC
***CASP9*-R**	TCACGGCAGAAGTTCACATTG
***GAPDH*-F**	TGCACCACCAACTGCTTAGC
***GAPDH*-R**	GGCATGGACTGTGGTCATGAG

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Cell viability assay

Cell viability of KG1 and M07e cells was assessed using the tetrazolium (MTT) colorimetric assay. According to MTT reduction carried out at three-time points after the start of the transfection. First, 200 µl MTT (Sigma-Aldrich, USA) with 50 mg/ml concentration was added to 5 $\times 10^5$ KG1 and M07e cells suspension in 2 ml RPMI 1640 medium and was then incubated 4 h at 37 ºC under 5% CO_2 in the darkness. Subsequently, 200 µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) was added to each well followed by shaking for dissolving formazan salt. The OD was measured at 570 nm by using a spectrophotometer (PG Instruments T80, PG Instrument, England). Cell viability was determined as absorption of the aliquot of transfected cells normalized to the absorption of control cells maintained in complete medium.

Statistical analysis

All tests were performed three times, and the data were analyzed using SPSS version 25 software. Also, GraphPad Prism version 8.3.0.538 was used to draw graphs. To evaluate differences between groups, data were analyzed by a two-way ANOVA test. The data are represented as mean ± with the production of water-insoluble purple formazan by mitochondrial dehydrogenase in the active cells, this method is straightly correlated with the number of living cells. The MTT assay was standard deviation (mean ± SD). For all statistical analyzes, P<0.001 was considered statistically significant.

Results

Antisense LNA GapmeRs decreased *SOX12* expression

The transfection efficiency of antisense LNA GapmeRs transfected cells was determined by fluorescence microscopy due to the oligonucleotide strands' FAM group (Figure 1). Additionally, flow cytometry confirmed the transfection level by more than 90% (data not shown). QRT-PCR was performed in two human acute myeloid cell lines, including KG1 and M07e, to measure the expression of *SOX12* in all groups (control, ALGNC, antisense LNA GapmeRs) at three-time points after transfection. *SOX12* expression was decreased dramatically in the antisense LNA GapmeRs group at all time points in comparison with other groups in both cell lines (P <0.001) (Figure 2a, 2b).

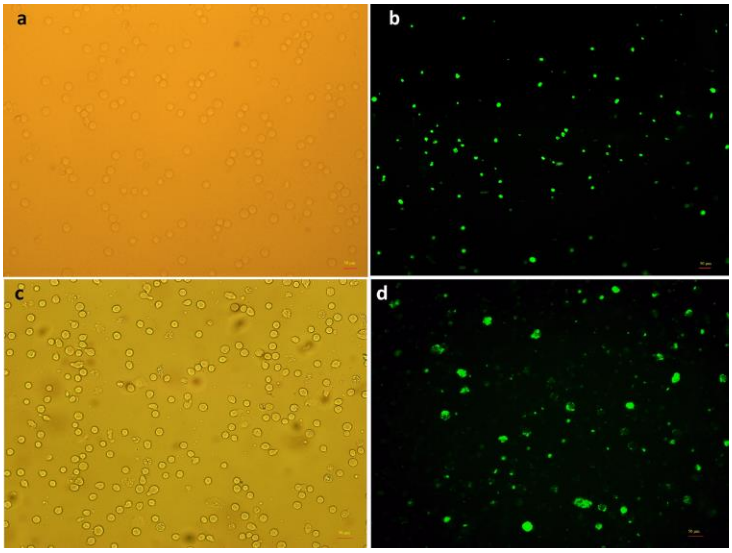

Fig. 1. Transfection of AML cell lines with antisense LNA GapmeRs. KG1 and M07e cells have been transfected with 6-FAM labeled antisense LNA GapmeRs. To evaluate the transfection efficiency of both cell lines, which have been assessed by a fluorescent microscope. a, b: phase contrast (a) and fluorescent (b) images of the same field of KG1 cells; c, d: phase contrast (c) and fluorescent (d) images of the same field of M07e cells. The majority of cells in both cell lines were transfected. Scale bars: 50 µm.

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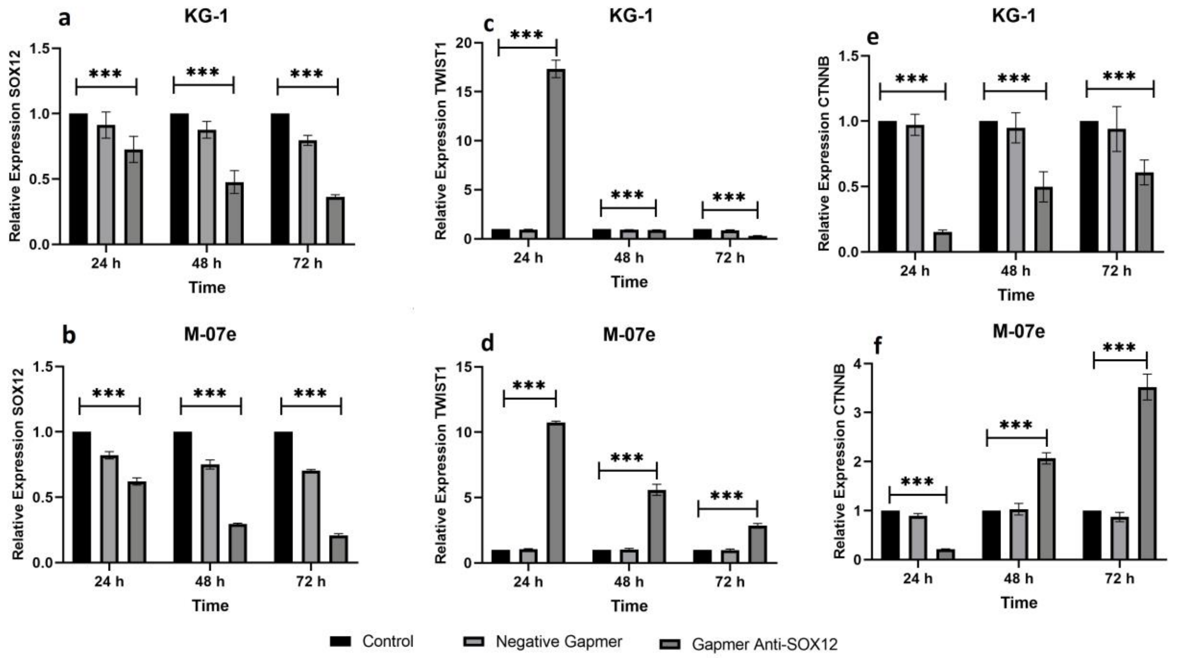

Fig. 2. Evaluation of *the* SOX12, *TWIST 1*, and *CTNNB1*expression in AML cell lines. a, b: expression of *SOX12* in KG1 (a) and M07e (b) cells; c, d: expression of *TWIST1* in KG1 (c) and M07e (d) cells ; e, f: expression of *CTNNB1* in KG1 (e) and M07e (f) cells. All genes expression assessment was performed by real-time PCR at 24, 48, and 72 h after GapmeR anti-*SOX12* transfection. Data analysis was performed by ΔΔCt method, and the untreated group (control) was reflected as a reference for comparison with other groups. The data are presented as mean ± SD of triple independent tests (***P < 0.001).

Knockdown of *SOX12* decreased the expression of *TWIST1*

The qRT-PCR assay was used to evaluate the effect of downregulation of *SOX12* on *TWIST1* expression in AML cell lines at 24, 48, and 72 h post-transfection. In all groups, gene expression of *TWIST1* was decreased considerably after transfection. The expression of *TWIST1* was gradually decreasing over time as its expression was at the lowest level 72 h post-transfection in KG1 (Figure 2c) and M07e (Figure 2d) (P<0.001). However, the downregulation of *TWIST1* in the KG1 cell line was more remarkable than its expression in the M07e cell line.

Assessment of *CTNNB1* expression in KG1 and M07e cell lines

The qRT-PCR assay was performed to determine the effect of downregulation of *SOX12* on *CTNNB1* expression in AML cell lines at 24, 48, and 72 h post-transfection. The results in KG1 cells demonstrated that *CTNNB1* expression was decreased in GapmeR anti-*SOX12* in comparison with the two other groups at each time, and its expression was at the lowest level 24 h after transfection (P<0.001) (Figure 2e). In M07e cells, although the most significant reduction was observed 24 h after transfection, *CTTNB1* expression increased gradually over time (P<0.001) (Figure 2f).

Expression level of *CASP3* and *CASP9* increa-sed after *SOX12* blockage

The qRT-PCR assay was designed to detect the expression level of *CASP3* and *CASP9* in KG1 and M07e in all groups at three-time points (24, 48, and 72 h). The *CASP3* and *CASP9* expression were upper in the antisense LNA GapmeR group in comparison with the control groups at all three time points in the KG1 cell line (P < 0.001) (Figure 3a, 3c) and in M07e cell line (P < 0.001) (Figure 3b, 3d). The highest level of *CASP3* was observed 72 h after transfection in the M07e cell line (Figure 3b). Also, *CASP9* was overexpressed 72 h after transfection in the M07e cell line (Figure 3d). Together, these results demonstrated that knockdown of *SOX12* significantly induced gene expression of apoptotic factors in AML cell lines.

Cell viability reduction in KG1 and M07e cells by inhibition of *SOX12*

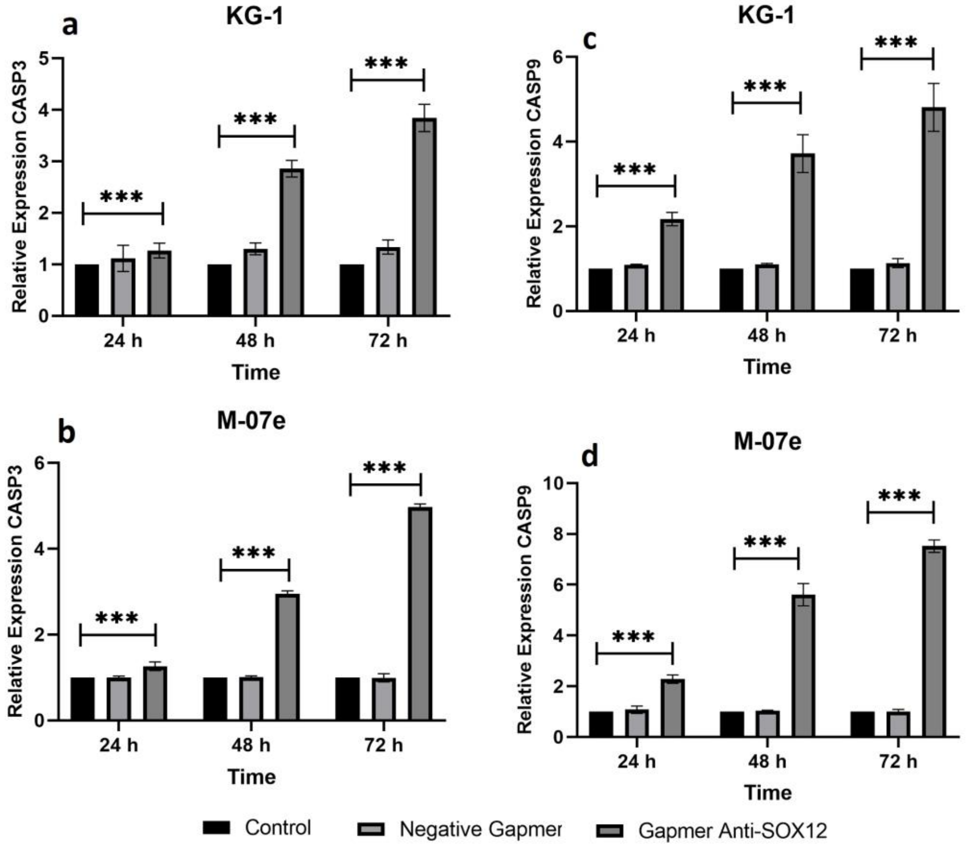

Fig. 3. Evaluation of *CASP3* and *CASP9* expression in AML cell lines. a, b) *CASP3* expression level in KG1 (a) and M07e (b) cell; c, d: *CASP9* expression level in KG1 (c) and M07e (d) cells. All genes expression assessment was performed by real-time PCR at 24, 48, and 72 h after GapmeR anti-*SOX12* transfection. Data analysis was performed by ΔΔCt method, and the untreated group (control) was considered as a reference for comparison with other groups. The data are displayed as mean ± SD of three independent experiments (***P < 0.001).

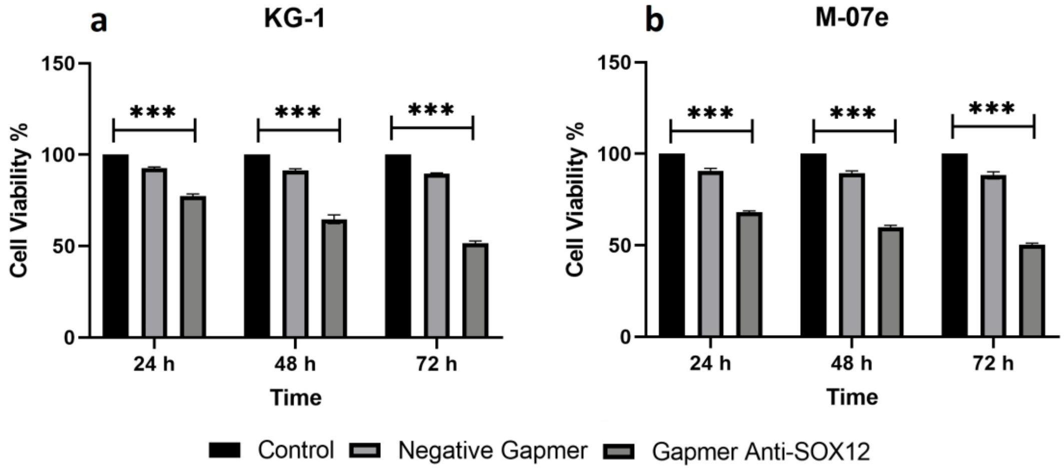

Fig. 4. AML cell lines viability after GapmeR anti-*SOX12* transfection. a) KG1 cells; b) M07e cells. Cells viability was measured by MTT assay at 24, 48, and 72 h after GapmeR anti-*SOX12* transfection. The viability of untreated cells (control group) at all three-time points has been considered 100%, and the viability of the other groups is represented as a percentage of untreated cells at the same time points. Data are mean ± SD of three independent experiments (***P <0.001).

The MTT test at 24, 48 and, 72 h after transfection was performed to investigate the effects of *SOX12* blockage on cell viability in KG1 and M07e cell lines. The cell viability in both cell lines was considerably decreased and was about 50% in 72 h after Gapmer anti-*SOX12* transfection. Compared to the control group, the viability in ALG and ALGNC groups was reduced, but the reduction in the ALG group was notably significant (Figure 4a, 4b). The ALGNC group's reduction may have been due to the toxicity of transfection reagents or the cells' senescence (P <0.001).

Discussion

This is the first study to specifically explore the *SOX12* inhibition with antisense LNA GapmeRs in AML. The results demonstrate that *SOX12* expression was decreased remarkably in the ALG group. Moreover, *SOX12* knockdown was associated with a decrease in *TWIST1* and *CTNNB1* expression. However, as mentioned in the results, maximum reduction for these two genes was observed at different time. *SOX12* knockdown could affect apoptotic factors such as *CASP3* and *CASP9*. Using the MTT assay, it was demonstrated that cell viability reduction was associated with the inhibition of *SOX12*. *SOX12* is one of the *SOXC* family members. It is suggested that *SOXC* genes have a vital role in redundancy to control cell differentiation and expansion (18). Several *SOXC* family members, such as *SOX4*, *SOX11*, and *SOX12*, have been overexpressed in human cancer tissues (15, 19, 20). *SOX12* has recently come into view as a novel oncogene. It is overexpressed in hepatocellular carcinoma with regional lymph nodes and remote metastases (21). Furthermore, *SOX12* is a potential cancer-stem-cell-like marker for hepatocellular carcinoma that increases chemoresistance (22). Different studies indicated that the downregulation of *SOX12* induces an antitumor effect in breast, lung, pancreatic, and colorectal cancer cells (19, 20, 23, 24). On the other hand, several studies were perfoemed on the association of *SOX* genes and AML (25, 26). In particular, a study illustrated that *SOX12* was highly expressed in AML in comparison with normal hematopoietic stem and progenitor cells, and its knockdown in THP1 cells or primary AML cells with a lentivirus containing shRNAs was shown to inhibit cell proliferation and reduce the clonogenicity and leukemia propagation (11). Interestingly, in a recent study, *SOX12* could have been blocked by antisense LNA GapmeR; thus, its expression decreased significantly.

To further confirm the transactivating of *SOX12*, we investigated *TWIST1* expression as well as *SOX12* expression as a direct transcriptional target of *SOX12*. It has been reported that the silencing of *SOX12* remarkably decreased the mRNA and protein expression of *TWIST1* in breast cancer cells. Moreover, the change in *TWIST*'s mRNA levels indicates that *SOX12* may bind to this factor's promoter to control its transcription (20). Another study has been shown that *SOX12* promotes migration and invasion of hepatocellular cacinoma through upregulating *TWIST1* and *FGFBP1* (15). Moreover, knockdown of *SOX12* in lung cancer cell lines, such as SPC-A-1 and A549 cells, led to a significant decrease in the mRNA and protein level of *TWIST1*. This study also demonstrated that *SOX12* directly binds to the promoters of cyclin E and *TWIST1* in A549 cells (19). Together with these studies, our data indicated that knockdown of *SOX12* in both KG1 and M07e cells led to a significant decrease in the mRNA expression of *TWIST1*. Several studies demonstrated that the functions of the *SOX* genes and WNT/CTNNB1 pathway correlate with the regulation of β-catenin expression (13). In this study, we investigated the association between knocking down *SOX12* and *CTTNB1* expression, especially in AML diseases. As a result, in both cell lines, *CTTNB1* expression decreased after 24 h, and gradually until 72 h, its expression increased, which

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can be due to the effect of other signaling pathways.

Apoptosis or programmed cell death is the primary mechanism for eliminating damaged, unwanted cells that cannot be restored (27). Two predominant signaling cascades have been delineated to initiate the apoptotic program in cells, the intrinsic (mitochondrial) and extrinsic (death ligand) pathways (28, 29). Both cascades lead to the activation of the executive caspase 3, 6, and 9, which are essential mediators of apoptosis (30). So, caspase plays a pivotal role in the executive phase and, its relevance in many cancer cells and cancer cell lines has been demonstrated (31-33). To investigate apoptotic factors' expression after the knockdown of *SOX12*, we determined the expression of *CASP3* and *CASP9*. As expected, these factors increased in both KG1 and M07e cell lines.

In conclusion, we revealed that *SOX12* expression and related genes, *TWIST1* and *CTNNB1*, were downregulated in AML cell lines. Moreover, we showed that *SOX12* plays a vital role in the programmed cell death of AML cell lines. Our results can be useful in translational medicine for subsequent research in AML treatment and generate cutting-edge drugs based on antisense therapy. This study suggested that *SOX12* may be a novel biomarker for AML, and can be translated into novel therapeutic strategies for AML.

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Conflict of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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