

Down-Regulated gga-miR-223 Inhibits Proliferation and Induces Apoptosis of MG-infected DF-1 Cells by Targeting FOXO3

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Abstract

Background

Mycoplasma gallisepticum (MG) is a major poultry pathogen that can induce Chronic Respiratory Disease (CRD) in chickens, causing serious economic losses in poultry industry worldwide. Increasing evidence suggests that microRNAs (miRNAs) act as a vital role in resisting microbial pathogenesis and maintaining cellular mechanism. Our previous miRNAs sequencing data showed that gga-miR-223 expression level significantly decreased in MG-infected chicken lungs. The aim of this study was to reveal the role of gga-miR-223 in MG-induced CRD progression.

Results

We found that gga-miR-223 was remarkably down regulated and forkhead box O3 (FOXO3) was up-regulated in both MG-infected chicken embryos lungs and the chicken embryonic fibroblast cell line (DF-1) by qPCR. FOXO3 was verified as the target gene of gga-miR-223 through bioinformatics analysis and dual-luciferase reporter assay. Further studies showed that overexpressed gga-miR-223 could promote cell proliferation, cell cycle, and inhibit cell apoptosis by notably promoting the expression of cell cycle marker genes cyclin-dependent kinase 1 (CDK1), cyclin-dependent kinase 6 (CDK6) and Cyclin D1 (CCND1) and inhibiting the expression of apoptosis markers Bcl-2-like 11(BIM), FAS ligand (FASLG) and TNF-related apoptosis-inducing ligand (TRAIL). As expected, FOXO3 knockdown group got similar results. Overexpression of gga-miR-223 observably promoted cell multiplication, cell cycle progression, and inhibited apoptosis of MG-infected DF-1 cells, while inhibited gga-miR-223 had the opposite effect.

Conclusions

Taken together, upon MG-infection, downregulated gga-miR-223 could decrease proliferation, cycle progression, and increase apoptosis through directly targeting FOXO3 to exert an aggravating MG-infectious effect.

Background

Chronic Respiratory Disease (CRD) is one of the most common chronic diseases in the global poultry industry. *Mycoplasma* is a kind of cell wall-free, self-reproduction, and intracellular parasitics. Mycoplasma is widespread all over the world and can invade a wide range of species such as human, dog, rabbit, chicken and all domestic animals, resulting in multiple diseases [1]. *Mycoplasma gallisepticum* (MG) is one of the most important pathogens in avian mycoplasma infection and frequently causes CRD in chickens. The host mounts a vigorous inflammatory response to MG, resulting in respiratory distress in chickens. Although vaccination and antibiotics can be used to control the infection, it is impossible to completely clear MG from infected chickens. MG-caused avian chronic respiratory disease has an increased epidemic, leading to tremendous economic losses in poultry industry [2]. MG-HS, isolated from a chicken farm in Hubei Province, was demonstrated to be a virulent

strain of MG through a series of physiological and biochemical reactions [3]. Nowadays, increasing attention is paid to Mycoplasma-induced CRD, which remains much to be explored [4-6].

MicroRNAs (miRNAs) are small, endogenous and non-coding RNAs with a length of 18-25nt, function as post-transcriptional regulators of their target mRNAs expression by binding to the 3'-untranslated region (3'-UTR) of their target mRNAs [7]. Recent studies indicated that miRNAs played essential roles in multiple biological processes, especially cell proliferation, apoptosis, migration and inflammation [8-11]. Additionally, it has been reported that miRNAs are involved in many major poultry diseases, such as avian influenza, infection bursal disease, and avian leucosis [12-15]. Our previous research has revealed that some miRNAs (gga-miR-99a, gga-miR-16-5p, gga-miR-451 and gga-miR-101-3p) significantly suppressed the proliferation of MG-infected DF-1 cells by promoted apoptosis [16-19]. However, it is largely unclear how miRNAs regulate MG infection. MiR-223 is highly conservative among different species and plays an important role in immune cell development, inflammation and other biological processes [11, 20]. MiR-223 could inhibit nicotinamide phosphoribosyl transferase (NAMPT) to regulate the proliferation and senescence of endothelial progenitor cells (EPCs) [21]; miR-223 inhibited cell proliferation and enhanced cell apoptosis in acute myeloid leukemia (AML) cell lines [22]. Numerous studies have revealed that miR-223 functions as a regulatory factor to assist the proliferation and invasion of diverse cancers, such as liver cancer[23], lung cancer [24] and breast cancer[25]. Even though current studies have shown that miR-223 plays an important role in immunity and various pathological responses[26], it still remains unclear how miR-223 is involved in the regulation of MG infection.

We found that gga-miR-223 was significantly down-regulated in MG-infected chicken embryo lungs by Solexa deep sequencing [27]. In the present study, we further validated that gga-miR-223 was remarkably down-regulated in MG-infected chicken embryo lungs and MG-infected DF-1 cells. FOXO3 was demonstrated as the direct target gene of gga-miR-223. Down-regulated of gga-miR-223 significantly inhibited the MG-infected DF-1 cells proliferation and promoted apoptosis by regulating CDK1, CDK6, CCND1, BIM, FASLG and TRAIL. This study suggested that down-regulated 223 inhibits cell proliferation and promotes apoptosis by targeting FOXO3 to facilitate MG infection.

Materials And Methods

Reagents and cell culture

The chicken DF-1 cells were purchased from Huiying (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Shanghai, China) in a carbon dioxide cell incubator with 5% CO₂ at 37 °C.

Mycoplasma Strains

MG-HS, a virulent strain of mycoplasma, was isolated from a chicken farm in Hubei Province, China [3]. MG-HS was presented by the State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University (Wuhan, China). MG-HS was cultured on the basis of previous

study report[28]. A color-changing unit (CCU) assay was applied to determine MG-HS viable number in suspension.

Infection Experiments

To study the MG infection mechanism, we established an experimental group and a blank group and started infection experiment. Meanwhile, DF-1 cells were cultured in six-well plates without any antibiotics. When cells covered 50-60% confluence, the experimental group was infected with MG-HS (1×10^{10} CCU/mL) about 130 μ L. After 24h infection, two group cells were collected with TRNzol Universal Reagent kit (TIANGEN, Beijing, China) for the follow-up experiment.

RNA Isolation and Quantitative Real-Time PCR

Total miRNA was isolated from post-infected and non-infected cells via TRNzol Universal Reagent kit (TIANGEN, Beijing, China), according to the manufacturer's instructions. Then, RNA, diluted to 1 μ g, was inverse transcribed to cDNA with the first strand cDNA synthesis kit (TaKaRa, Tokyo, Japan) and reverse transcription PCR (RT-PCR). RT-qPCR was performed to detect the expression of gga-miR-223 (normalized to 5s-RNA), FOXO3 (normalized to GAPDH) through the RT-PCR machines (Bio-Rad, Hercules, CA, USA) with SYBR-green (Tiangen, Beijing, China). Each sample was repeated least three times.

DNA Primers and RNA Oligonucleotides

In this experiment, total sequences of DNA primers that was adopted are presented in Table 1. In addition, All RNA oligonucleotides designed and synthesized by GenePharm (Shanghai, China).

Table 1. Sequences of DNA primers.

Name	Primer Sequence (5'-3')
Primers for 3'-UTR Cloning	
FOX03 3'UTR-F	CCCTCGAGAAATGGGCAAACAGGCCCTC
FOX03 3'UTR-R	TTGCGGCCGCATGCAGGGTTCACAAACGTC
Mut-FOX03-3'UTR-F	CAGGCCCTCATTGACTGTCGAGATGTAGAGAGAGAACCCTTTGCC
Mut-FOX03-3'UTR-R	CTCTGTAGGTCTTGACAGTCAAAGAGGGTCTGCTTGCCTCTTCCC
Primers for RT-qPCR	
GAPDH-F	GAGGGTAGTGAAGGCTGCTG
GAPDH-R	CACAACACGGTTGCTGTATC
FOX03-F	CGGACAAATTCGAACGCCAG
FOX03-R	GCACGGTTTGTTTACCGAGG
RT-gga-miR-223-F	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGGGTATT
gga-miR-223-F	CTGGTAGGTGTCAGTTTG
gga-miR-223-R	ACTGGTGTCGTGGAGTCGGC
gga-5s-rRNA-F	CCATACCACCCTGGAAACGC
gga-5s-rRNA-R	TACTAACCGAGCCCGACCCT
<i>CDK1</i> -F	TGGGAACGCATGTCCAAAAC
<i>CDK1</i> -R	GCAGGCAGGCAAAGATAAAGAC
<i>CDK6</i> -F	CACCCCAACGTGGTCAGATT
<i>CDK6</i> -R	GGCCACATCATTAGGCCAGT
<i>CCND1</i> -F	GACTTTTGTGGCTCTGTGCG
<i>CCND1</i> -R	CTGTTCTTGGCAGGCTCGTA
<i>BIM</i> -F	CTTCTTCTTCGTGCGGAGGT
<i>BIM</i> -R	TCTGCAAGCGAGTGAGATCG
<i>FASLG</i> -F	CTCCCATTTGGAGTGGGAACC
<i>FASLG</i> -R	CTTCCCCGGAAGAGCACATT
<i>TRAIL</i> -F	CAGAACTTGGACGTGGTGGA

Prediction of gga-miR-223 Target Genes

Bioinformatic analysis, TargetScan (<http://www.targetscan.org/>) and the other is miRDB (<http://www.mirdb.org/miRDB/>), were used to find which gene was the most potential target. The gene prediction scores and conservation degree as well as experimental goals were taken into account. Besides, it meant qualified that the prediction score was 60 and perfect that the prediction score was 100. RNA secondary structure between gga-miR-223 and the 3'-UTR of its target gene was analyzed by RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>).

Construction of 3'-UTR-luciferase Plasmid and Dual-luciferase Reporter Assay

For dual-luciferase reporter assay, we had designed wide-type (WT) and mutant 3'-UTR primers of FOXO3 and obtained corresponding DNA fragment which covered the predicted gga-miR-223 binding site. Relying on Xho I/Not I nuclease, Psi-CHECK™-2(Promega, Madison, WI, USA), Luciferase vector, was combined with FOXO3 3'-UTR (wild-type and mutant) to construct psiCHECK-2-FOXO3-3'-UTR (wild-type and mutant) vector.

DF-1 cells were cultured in 24-well plates at 2×10^5 cells or so per well for the luciferase assay. When cells reached 80-90% confluence, using Lipofectamine 3000 (Invitrogen Life Technologies, USA) each co-transfected cells with wildtype or mutant reporter plasmid (200 ng) and 10 pmol of the indicated RNA oligonucleotides, including gga-miR-223 and gga-miR-223-NC. DF-1 Cells were harvested after transfection for 48 hours. Next, the luciferase activity in each group was detected by using automatic microplate reader (Bio-Rad, Hercules, CA, USA) in accordance with the dual luciferase reporter gene detection kit instructions (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Overexpression and Inhibition of gga-miR-223

To explore the effects of overexpressed and inhibitory gga-miR-223 on FOXO3, we conducted a series of experiments to detect FOXO3 mRNA relative expression under different circumstances where gga-miR-223 was overexpressed or inhibited. DF-1 cells were cultured in 6-well plates. Once reached 80-90% confluence, each group cells would be transfected separately with gga-miR-223, gga-miR-223-NC, gga-miR-223-Inh and gga-miR-223-Inh-NC. 48h after transfection, TRNzol was used to harvest total cells. Subsequently, we had extracted total RNA and performed reverse transcription experiment. Next, the expression levels of gga-miR-223 and FOXO3 in each group were detected by qPCR. Meanwhile, 5S-RNA and GAPDH served as an international control.

Cell Proliferation and Apoptosis Assays

The Cell Counting Kit-8(CCK-8) Kit was used for growth curve experiments. Cell suspension was prepared from logarithmic growth phase cells and the DF-1 cells were then diluted and inoculated on a 96-well plate at a density of 2×10^4 cells per well. At 39 °C with 5% CO₂ DF-1 cells were cultured to stick to the wall. Next, MG-HS was utilized to infect DF-1 cells for 2 hours. Each group cells were separately transfected gga-miR-223 mimics, NC, inhibitor and inhibitor NC using LipofectamineTM3000 and each group had 6 biological replicates. Meanwhile, a blank group without any RNA oligonucleotides was also set up. At 24h, 48h, and 72 h post-transfection, 10 ul of CCK-8 solution were added to each well of the cells to be tested and all cells continued to incubate in a cell incubator for 1-4 hours. Subsequently, absorbance at a 450 nm wavelength was measured on a microplate reader (Bio-Rad, USA).

Made from logarithmic growth phase DF-1 cells, Cell suspension was infected MG-HS for 2 hours and then separately transfected RNA oligonucleotides according to the instruction of LipofectamineTM3000. At 48h post-transfection, cold-ice phosphate-buffered saline (PBS) was utilized to wash cells once, harvested cells with the centrifuge (2000 r/min, 5 min) and resuspended in binding buffer containing 5µl Annexin V-FITC at a density of $1 - 5 \times 10^5$ cells/mL. DF-1 Cells were then incubated with 5 µl propidium iodide (PI) for 15 min in the dark. Finally, the apoptosis of DF-1 cell was analyzed by fluorescence-activated cell sorting (BD Biosciences). Each group was repeated three times.

Statistical Analysis

In this study, all data were presented as the mean values \pm SDs of at least three independent experiments. The one-way ANOVA with the Duncan's multiple-range test and Student's t-test were to evaluate statistically significant differences. $P < 0.05$ was considered significant. Significant differences were performed as * $p < 0.05$ and ** $p < 0.01$.

Results

Gga-miR-223 is Remarkably Suppressed in MG-Infected DF-1 Cells and chicken embryonic lung tissue

In the previous study, we found that gga-miR-223 was significantly down-regulated in chicken embryonic lungs with MG infection by miRNAs deep sequencing [29]. The expression level of miR-223 in chicken embryo lung tissues and cells were further verified by qPCR. The results showed that compared with normal lung tissue, gga-miR-223 was down-regulated ($p < 0.05$) after 4 and 6 days of infection, and on the 8th day of infection, the expression level of gga-miR-223 was highly down-regulated ($p < 0.01$) (Figure. 1A). Meanwhile, gga-miR-223 was remarkably suppressed in DF-1 cells with MG infection compared with control group (Figure. 1B), which was consistent with the sequencing results. Therefore, these results imply that gga-miR-223 may be involved in MG infection.

Figure 1. Relative gga-miR-223 expression level in MG-infected DF-1 cells and chicken embryonic lung tissue. (A) On the 9th day of the chicken embryo hatching, 130µLMG-HS was injected into the allantoic fluid, and the expression of gga-miR-223 was measured by qPCR on the 4th, 6th, and 8th day after the challenge. (B) DF-1 cells were cultured in 6-wells plate and treated MG. After 24h infection, the total RNA was

isolated from infected cells via TRNzol Universal and the gga-miR-223 expression was detected by RT-qPCR. 5s-RNA was used as the internal quantitative control gene. All measurements shown were the means \pm SD from three independent experiments, each with three replicates. (*, $P < 0.05$;**, $P < 0.01$)

miR-223 is able to bind to the 3'-UTR of FOXO3 mRNA

It is well known that miRNAs exert a role in decreasing and inhibiting transcriptional gene level by binding target miRNAs, the expression of their made a difference by degrading corresponding target genes. TargetScan (<http://www.targetscan.org>) and miRDB (<http://www.mirdb.org/miRDB/index.html>) were utilized to predict the potential target genes of gga-miR-223. FOXO3 was chosen the best appropriate target gene within 107 potential targets with the help of Gene Scoring system, and the highly conserved target sequence from position 42 to 49bp in different species. (Figure. 2A).

RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>) was used to analyze the minimum free energy (mfe) and space molecular structure when gga-miR-223 combined with FOXO3 3'-UTR. The mFE is about -22.2 kCal/mol, which indicated high stability (Figure. 2B).

To further verify whether gga-miR-223 could combine with FOXO3 3'-UTR, we performed the dual-luciferase reporter assay by transfecting the luciferase reporter vectors containing either the wild-type or the mutant 3cifer of FOXO3 into DF-1 cells. The transfection of gga-miR-223 mimics significantly decreased the luciferase activity of the reporter containing wild-type 3'-UTR compared with the control, but there was no significant effect of the reporter containing mutant 3'UTR (Figure 2C, D). These results showed that gga-miR-223 inhibited FOXO3 expression by directly binding to its complementary sequence in the 3'-UTR of FOXO3.

Figure 2. (A, B) Prediction and analysis of gga-miR-223 target genes. FOXO3 was identified as an appropriate candidate. (A) The conservation degree of target sequences among different species. FOXO3 3'-UTR from various species all contained highly consistent seed sequence. Position 42–49 in the 3'-UTR of FOXO3, which is highlighted, was predicted to be the target site of it. The seed sequence in gga-miR-223 is also highlighted. (B) The minimum free energy (mfe) and space molecular structure when gga-miR-223 combined with FOXO3 3'-UTR. (C, D) FOXO3 was a direct target of gga-miR-223 in DF-1 cells. (C) The putative wild-type FOXO3 3'-UTR binding sequences in gga-miR-223, but the mutation sequence of FOXO3 3'-UTR was not matched with the gga-miR-223. (D) A dual-luciferase reporter assays were used to assess the effect of gga-miR-223 on FOXO3; DF-1 cells were separately transfected with wild-type or mutation luciferase vectors as well as the indicated RNA oligonucleotides. After 48h transfection, collecting all cells and measuring fluorescence. Bars that do not share the same letters are significantly different ($p < 0.05$) from each other. The data was expressed as the mean \pm SD of triplicate cell cultures.

FOXO3 expression is negatively regulated by gga-miR-223

In order to further explore the function of gga-miR-223, we examined whether gga-miR-223 could regulate the expression of FOXO3 in DF-1 cells. The DF-1 cells transfected with gga-miR-223 mimics were marked

as miR-223; cells transfected with a non-specific RNA (marked as miR-223-NC); cells transfected with gga-miR-223 inhibitor (marked as miR-223-Inh). We found that, whether at the mRNA level or at the protein level, the over-expression of gga-miR-223 significantly suppressed the expression of FOXO3 in DF-1 cells, whereas miR-223-Inh had the opposite function (Figure 3A, 3B). These results indicate that FOXO3 is a direct target of gga-miR-223 in DF-1 cells and its expression is negatively regulated by gga-miR-223.

Figure 3. Gga-miR-223 negatively regulates FOXO3 expression. DF-1 cells were transfected with miR-223-mimics, miR-223-inhibitor and miR-223NC, respectively, and the relative expression of FOXO3 mRNA and protein was detected by qPCR and Western-blotting. (A) Detection of relative expression of FOXO3 mRNA in DF-1 cells by qPCR. GAPDH served as an international control. (B) The protein levels of FOXO3 in different treatment groups were detected by Western-blotting. The FOXO3 protein expression was normalized to GAPDH. All values shown were the means \pm SD from three independent experiments, each with three replicates. (**, $P < 0.01$)

MG-infection up-regulate FOXO3 expression

As mentioned above, FOXO3 was the direct target gene of gga-miR-223 and the expression of gga-miR-223 was remarkably down-regulated in DF-1 cells with MG infection. In order to further determine the effect of MG infection on FOXO3, we detected the expression of FOXO3 in MG-infected chicken embryos and DF-1 cells. On days 4, 6, and 8 post-infection, the expression level of FOXO3 were remarkably up-regulated ($p < 0.05$) (Figure 4A), which were opposite to the expression of gga-miR-223. In MG infected DF-1 cells, as we expected, the expression of FOXO3 was very significantly up-regulated at both mRNA level and protein level ($p < 0.01$) (Figure 4B, 4C).

Figure 4. MG-infection up-regulate FOXO3 expression. (A) 130 μ L MG-HS was injected into allantoic fluid on the 9th day of chicken embryo hatching. The lung tissue of chicken embryo was taken on the 4th, 6th and 8th day after challenge, and the relative expression of FOXO3 in the lung tissue was detected by qPCR. (B) DF-1 cells were assessed by RT-qPCR. The noninfected group serves as negative control. The relative expression of FOXO3 was normalized to GAPDH. (C) The protein levels of FOXO3 in different treatment groups were detected by Western-blotting. The FOXO3 protein expression was normalized to GAPDH. All values shown were the means \pm SD from three independent experiments, each with three replicates. (*, $P < 0.05$; **, $P < 0.01$)

Gga-miR-223 promoted the proliferation of MG-HS infected DF-1 cells

As we all know, MG-HS infection can inhibit cell proliferation and promote apoptosis [30]. To investigate the potential effect of gga-miR-223 on cell proliferation, the DF-1 cells transfected with miR-223 mimics, miR-223-Inh, or miR-223 NC before MG infection; and non-transfected cells were marked as miR-free, all above groups were infected with MG. Uninfected DF-1 cells were marked as blank (MG-). The CCK-8 kit was used to detect the proliferation of DF-1 cells. The results showed that there was no significant difference in each group after 24h post-transfection. At 48h post-transfection, the number of cells in all MG infection groups were very observably lower than that in the blank (MG-) group ($p < 0.01$) (Figure 5);

compared with miR-223-NC (MG+) group, miR-free (MG +) group and miR-223 inhibitor (MG+), the number of cells in miR-223-mimics (MG+) group was significantly increased ($p < 0.05$) (Figure 5); but the number of cells in miR-223-inhibitor (MG+) group was markedly lower than that in other groups ($p < 0.01$); after 72 hours, although the number of cells in the miR-223-mimics (MG+) group was not different from that in the blank (MG-) group, it was significantly higher than the other groups ($p < 0.05$) (Figure 5). Data indicates that the presence of gga-miR-223 can resist the cell reduction caused by MG infection, thereby promoting the proliferation of DF-1 cells infected with MG.

Figure 5. Over-expression of gga-miR-223 promotes DF-1 cell proliferation. Cells were cultured in 96-well plates, DF-1 was divided into 5 groups and transfected with different RNA sequences (namely, miR-223 mimics (miR-223-mimics(MG+)) miR-223 inhibitor (miR-223-inhibitor(MG+)) miR-223 NC (miR-223-NC(MG+)) MG challenge control group (miR-free(MG+)) and blank control group (blank(MG-)), and challenged with MG-HS at 24h. Then CCK-8 kit was used to detect cell proliferation at different times. Data was expressed as the mean \pm SD of three independent experiments, each with three replicates. (*, $P < 0.05$; **, $P < 0.01$)

Gga-miR-223 promotes cell proliferation by affecting the cell cycle

Based on the results of the previous step, we also used flow cytometry to explore whether gga-miR-223 affects the cycle of MG-infected cells. In DF-1 cells, MG infection obviously increased the proportion of cells staying in G1-phase, while the proportion of S- and G2-phase cells distinctly decreased; unsurprisingly, overexpression of gga-miR-223 could remarkably improve the cell cycle changes caused by MG infection, significantly reduce the proportion of cells stuck in G1-phase and increase the proportion of cells in S- and G2-phase; and the effect of gga-miR-223-inhibitor transfection on cell cycle was exactly the opposite to that of gga-miR-223-mimics transfection (Figure 6). Taken together, these results suggested that MG infection made a large number of DF-1 cells stagnant in G1-phase, but overexpression of gga-miR-223 could effectively increase the transition from G1-phase to S- and G2-phase to promote cell growth and proliferation.

Figure 6. Overexpression of gga-miR-223 transformed DF-1 cells from G1-phase to S- and G2-phase. Cells were cultured in 96-well plates, DF-1 was divided into 5 groups and transfected with different RNA sequences (namely, miR-223 mimics (miR-223-mimics(MG+)) miR-223 inhibitor (miR-223-inhibitor(MG+)) miR-223 NC (miR-223-NC(MG+)) MG challenge control group (miR-free(MG+)) and blank control group (blank(MG-)), and challenged with MG-HS at 24h. Then CCK-8 kit was used to detect cell proliferation at different times. At 48 h post-transfection, the cell phase distribution was analyzed by flow cytometry. Bars that do not share the same letters are significantly different ($p < 0.05$) from each other. The data was expressed as the mean \pm SD of triplicate cell cultures.

Gga-miR-223 inhibits the apoptosis of MG-HS infected DF-1 cells

To further explore how miR-223 promotes DF-1 cells proliferation, the cell apoptosis was assayed by flow cytometer. Similarly, the synthetic RNA oligonucleotides were transfected into DF-1 cells and then co-

cultured with MG-HS for 48h. Cell apoptosis results showed that the apoptosis rate of blank (MG-) group was significantly decreased compared with other challenge groups ($p < 0.05$). The apoptosis rate of MG-infected cells with gga-miR-223 mimics was remarkably decreased compared with the miR-223-NC(MG+) group or miR-free (MG+) group, whereas the effect of Inhibitor (MG+) group was opposite ($p < 0.05$) (Figure 7).

Figure 7. Effects of overexpression and inhibition of gga-miR-223 on the cell apoptosis of DF-1 cells with MG infected. The cell apoptosis of DF-1 cells was remarkably suppressed by overexpress of gga-miR-223. The cell apoptosis of DF-1 cells was promoted by inhibition of gga-miR-223. Bars that do not share the same letters are significantly different ($p < 0.05$) from each other. All data from the triplicate experiments carried out independently were adopted as mean value \pm SD.

Gga-miR-223 promotes cycle gene expression and inhibits apoptosis gene expression

We have known that gga-miR-223 could regulate the cycle of MG-infected cells, promote their proliferation and inhibit their apoptosis. In order to further explore the details of the role of gga-miR-223, the expressions of genes which related to cell cycle and apoptosis were measured after transfection of synthetic RNA oligodeoxynucleotides in DF-1 cells. RT-qPCR detection showed that the relative expression of CDK1, CDK6 and CCND1 in miR-223-mimics group was dramatically higher than that in miR-223-inhibitor group and miR-223-NC group, while the relative expression of CDK1, CDK6 and CCND1 in miR-223-inhibitor group was significantly lower than that in miR-223-NC group ($p < 0.05$) (Figure 8A). These results indicate that the low expression of gga-mir-223 inhibits the cell cycle progression by inhibiting the expression of CDK1, CDK6 and CCND1.

The RT-qPCR detection of apoptotic genes showed that the relative expression of BIM, FASLG and TRAIL in miR-223-mimics group was notably decreasing than that in miR-223-inhibitor group and miR-223-NC group ($p < 0.05$), while the relative expression of FASLG, BIM and TRAIL in miR-223-inhibitor group was prominently increasing than that in miR-223-NC group ($p < 0.05$) (Figure 8B). The data proved that low-expressed gga-miR-223 promoted apoptosis by promoting the expression of BIM, FASLG and TRAIL.

Figure 8. Effects of gga-miR-223 on the cycle and apoptosis genes in DF-1 cells. The DF-1 cells transfected with gga-miR-223 mimics were marked as miR-223; cells transfected with a non-specific RNA were marked as miR-223-NC; cells transfected with gga-miR-223 inhibitor were marked as miR-223-Inh. After 24 hours of transfection of DF-1 cells to synthesize RNA oligonucleotides, RT-qPCR was used to detect the relative expression of marker genes mRNA. (A) Relative mRNA expression of cell cycle marker genes CDK1, CDK6 and CCND1. (B) Relative mRNA expression of apoptosis marker genes BIM, FASLG and TRAIL. Data was expressed as the mean \pm SD of three independent experiments, each with three replicates. (*, $P < 0.05$; **, $P < 0.01$)

Knockdown of FOXO3 promoted cell cycle genes expression and inhibited apoptosis genes expression

We have known that gga-miR-223 could promote cell proliferation by promoting the expression of cycle genes and inhibiting the expression of apoptotic genes in DF-1 cells. FOXO3, the direct target gene of gga-miR-223, its knockdown was used to further investigate the role of gga-miR-223 in DF-1 cells. The si-FOXO3 and siRNA-NC were transfected into DF-1 cells, and the relative expression of FOXO3 mRNA and protein were detected by RT-qPCR and Western-blotting. The results of qPCR and Western-blotting were consistent, that is, the expression of FOXO3 in FOXO3 knockdown group was much lower than that in siRNA-NC group ($P < 0.05$) (Figure 9A, 9B). It showed that the FOXO3 gene was successfully knocked down in DF-1 cell.

Based on the knockdown of FOXO3 gene, we measured the expression of cycle marker genes and apoptosis marker genes in DF-1 cells. As expected, the relative expressions of CDK1, CDK6 and CCND1 in si-FOXO3 group were observably higher than that in siRNA-NC group ($p < 0.05$), while the relative expression of FASLG and TRAIL were markedly lower than that in siRNA-NC group ($p < 0.01$). And compared with siRNA-NC group, the relative expression of BIM in the si-FOXO3 group was extremely significantly reduced ($p < 0.01$) (Figure 9C). Therefore, these results indicate that the up-regulated gga-miR-223 negatively regulated the expression of FOXO3, thereby inhibiting the expression of cycle genes and promoting the expression of apoptotic genes, to achieve the effect of affecting cell proliferation, cycle and apoptosis.

Figure 9. Effect of knock-down of FOXO3 in DF-1 on cell proliferation. 6-well plates were used to culture cell. When the cell density reached about, si-FOXO3 and siRNA-NC were transfected respectively. The relative expression of mRNA was measured by qPCR at 24 hours after cell transfection; and the relative expression of FOXO3 protein was detected by Western-blotting at 72 hours after transfection. (A) The mRNA level of FOXO3 in DF-1 cells. (B) Western blot analysis of FOXO3 protein expression in DF-1 cells. And the expression of GAPDH served as a loading control. (C) The relative expression of cell cycle and apoptosis genes after knocking down FOXO3 gene in DF-1 cells. Data was expressed as the mean \pm SD of three independent experiments, each with three replicates. (*, $P < 0.05$; **, $P < 0.01$)

Discussion

The CRD induced by MG, a worldwide avian pathogen, has caused huge economic loss, making it a hot topic in poultry research [31, 32]. MG can activate inflammatory response in the respiratory tract of chickens [33]. Notably, it is extremely contagious and easy to induce prevalence of other diseases such as influenza A virus [34]. Therefore, how to solve the problem of MG infection is particularly important. Previously, a large number of researches indicated that pathogenic microorganisms could induce the differential expression of some host miRNAs, regulating the expression of numerous target genes by binding to them and affecting the corresponding cellular behaviors and metabolism processes [35, 36]. Hence, it will be crucial to increase our understanding of MG infection mechanisms by studying the potential role of miRNAs involved in MG infection.

Based on the previous studies, we found that *MG* could significantly regulate the expression of multiple miRNAs in the lung tissue of chicken embryo [27], suggesting that these differentially expressed miRNA might be effective markers for the diagnosis and prognosis and potential therapeutic interventions of MG-induced CRD. Previous high-throughput sequencing data showed that gga-miR-223 was signally down-regulated in *MG*-infected chicken embryo lung [37]. Importantly, accumulating data has suggested that miR-223 plays an important role in various diseases, especially respiratory disease [20, 38, 39]. MiR-223 plays a key role in regulating the immune inflammatory response to neutrophil asthma by inhibiting the NLRP3/ IL-1 β [40]; pharmacological blockade of the miR-223-NLRP3-IL-1 β signaling axis alleviates acute respiratory distress syndrome [41]. In addition, the expression level of miR-223 has dramatically altered by other bacterial infections, For instance, gastric inflammation caused by *Helicobacter pylori* infection through induction of miR-223-3P expression in a *Helicobacter pylori* CagA-dependent manner [42]; the expression of miR-223 in the breast tissue of dairy cows with *Streptococcus agalactis* mastitis increased three-fold, which may be involved in the signal pathway of *Streptococcus agalactis*-induced mastitis[40]. It indicates that miR-223 is strongly related to microbial infection. Consistent with these results, we discovered that gga-miR-223 was notably down-regulated in chicken embryonic lung tissue and DF-1 cells infected with MG (Figure 1), which indicated that gga-miR-223 was likely to be involved in the regulation of MG infection mechanism.

It is documented that miRNAs usually exert their regulatory effect the negative regulation of target genes at post-transcriptional level. There is innumerable evidence that miR-223 regulates cell physiological process by targeting FOXO3[43, 44]. Here, we have also identified FOXO3 as a target gene of gga-miR-223 by bioinformatic analysis and dual-luciferase assay (Figure 2). FOXO3 is a member of forkhead O (FOXO) transcription factors family, which has crucial roles in cell proliferation, cell cycle and apoptosis [45]. Many studies have shown that the up-regulated expression of FOXO3 in cells is associated with many diseases. FOXO3 is up-regulated in the cytoplasm of inflammatory colonic epithelial cells, leading to intestinal inflammation [46]; and up-regulated FOXO3 plays a key role in the migration of esophageal cancer cells [47]. Coincidentally, here, the expression of FOXO3 in MG-infected lung tissues and DF-1 cells also increased significantly (Figure 4), which is contrary to gga-miR-223, indicating that DF-1 cells infected with MG promote the expression of FOXO3 by downregulating gga-miR-223, thus presenting cellular physiological and biochemical changes.

In the process of MG infection, exogenous DNA and components from mycoplasma cause DNA damage to host cells and affect cell function, leading to apoptosis and inhibiting cell proliferation and cell cycle [48, 49]. MiR-223 has an effect on the proliferation and apoptosis of colorectal cancer cells by regulating FOXO3/BIM [50]. Some studies have shown that FOXO3 inhibits the proliferation of chicken liver cancer cell line (LMH) and induces its apoptosis [51]. It also has demonstrated that miR-223 directly suppresses FOXO3, and FOXO3 plays a critical role as a mediator of the biological effects of miR-223 in macrophage apoptosis [52]. Our results suggested that the presence of gga-miR-223 could resist the cell reduction caused by MG infection (Figure 5); overexpression of gga-miR-223 could effectively promote cell proliferation (Figure 6); and inhibit apoptosis rate of MG-infected cells with gga-miR-223 mimics was remarkably decreased (Figure 7).

CDKs are the core of cell cycle regulation, and Cyclins have a positive regulatory effect on CDKs ; BIM, FASLG and TRAIL are classic downstream genes of apoptosis [53], which suggest that abnormal expression of them contributes cell cycle arrest or apoptosis. In liver cancer cell, the translocated FOXO3 can directly promote the transcription of cyclin-dependent kinase inhibitors (CDKIs) p21Cip1 and p27Kip1, and on other hand can inhibit the expression of Skp2, leading to cell arrest in the G0/G1-phase [51]. FOXO3 can induce apoptosis by promoting TRAIL and inhibiting Bcl-2 expression. What's more, the phosphorylation of FOXO3 can be inhibited by casein, which reduces the expression of FOXM1 and its downstream gene CDK1, remarkably inhibiting the cell viability and cell growth. FOXO3 induced by mannitol can observably reduce the expression of CCND1 and CDK6, arresting cells in G1 phase, and inhibiting breast cancer cell cycle and activity. We found that, in this manuscript, cycle genes (CDK1, CDK6, CCND1) were inhibited and apoptosis genes (BIM, FASLG, TRAIL) were promoted in cells which treated with gga-miR-223 mimics or si-FOXO3 (Figure 8, 9). In general, it is now becoming clear that MG can regulate gga-miR-223 expression to affect the expression of FOXO3, and the influence cell proliferation and apoptosis. Therefore, we have sufficient reasons to speculate that MG regulates FOXO3 gene by changing the expression of gga-miR-223 to achieve regulation of cell proliferation and apoptosis.

Conclusions

In conclusion, when responding to MG infection, down-regulation of gga-miR-223 can increase the mRNA expression of cycle genes or decrease the mRNA expression of apoptosis genes, inhibit cell proliferation and promote cell apoptosis by targeting FOXO3. Therefore, the regulation of gga-miR-223 may provide a new therapeutic target for the CRD induced by MG in poultry.

More importantly, because of the high conservation of miR-223 and FOXO3 among different species (Figure. 2A), thus the results which obtained from chickens in this study will provide available information and insights for disease study of other species.

Abbreviations

MG: *Mycoplasma gallisepticum*; gga-miR-223: *Gallus gallus* microRNA-223; FOXO3: forkhead box O3; DF-1: the chicken embryonic fibroblast cell line; CDK1: cell cycle marker genes cyclin-dependent kinase 1; CDK6: cyclin-dependent kinase 6; CCND1: Cyclin D1; BIM: Bcl-2-like 11; FASLG: FAS ligand; TRAIL: TNF-related apoptosis-inducing ligand; 3'UTR: 3'-untranslated region.

Declarations

Acknowledgments

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Authors' contributions

X.P. conceived, designed and supervised the project. X.Y. and Y.W. performed experiments and wrote the manuscript. M.Z. helped to write, revise, and submit the manuscript. Y.H. and H.L. provided advices for the study. R.L. analyzed the data. Y.S. helped to revise the discussion.

Availability of data and materials

All data and analysis in the manuscript, if necessary, can be obtained from the corresponding author on reasonable request.

Ethics approval and consent to participate

These studies were approved by Huazhong Agriculture University Animal Research Ethics Committee (Wuhan, Hubei, China).

Consent for publication

Not applicable.

Competing Interests

The authors have declared that no competing interest exists.

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Figures

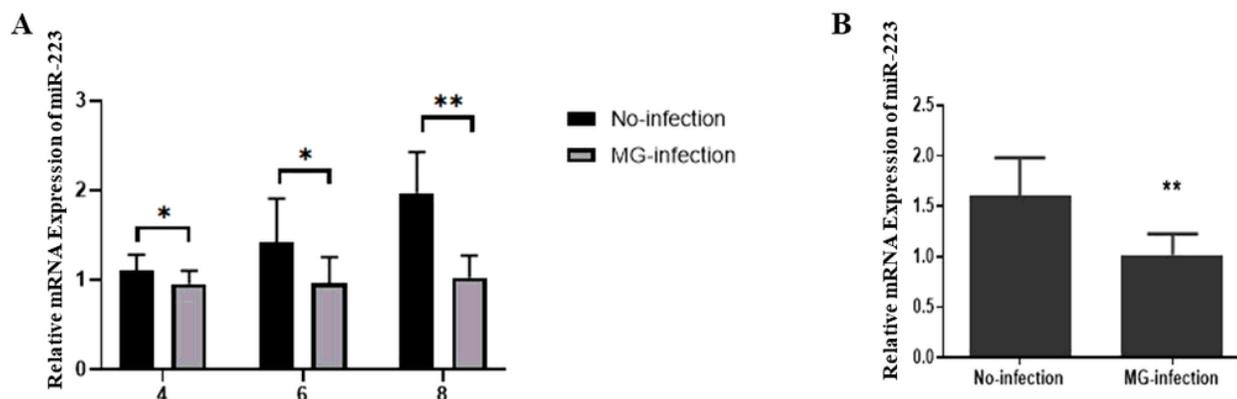


Figure 1

Relative gga-miR-223 expression level in MG-infected DF-1 cells and chicken embryo lung tissue.

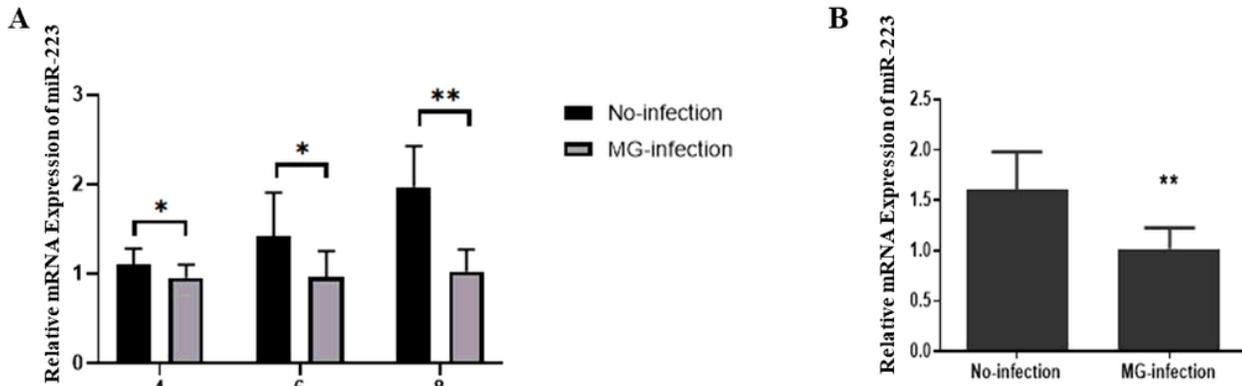


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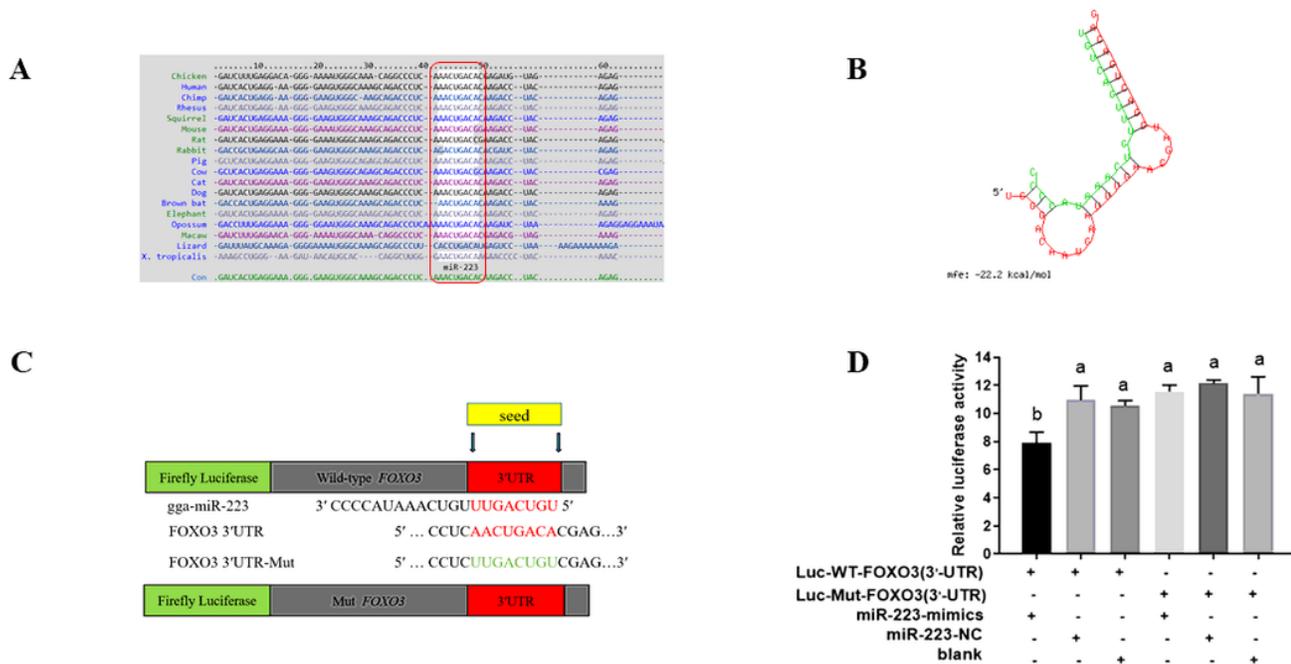


Figure 2

(A, B) Prediction and analysis of *gga-miR-223* target genes. *FOXO3* was identified as an appropriate candidate.

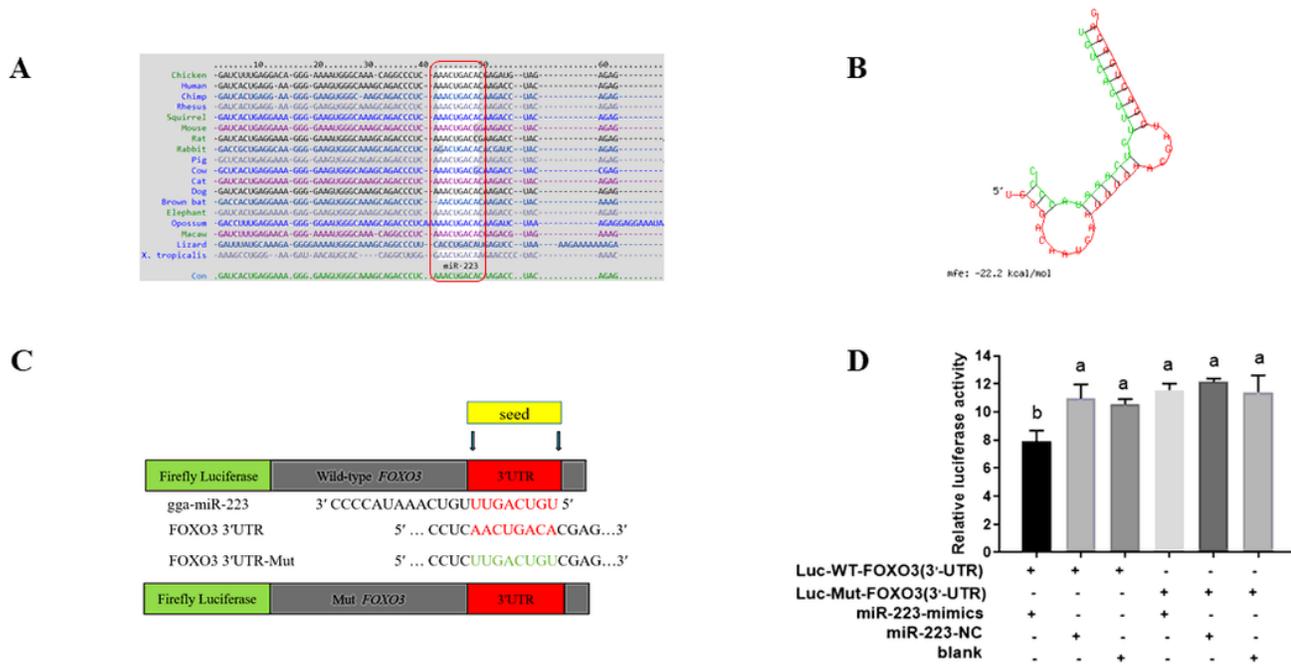


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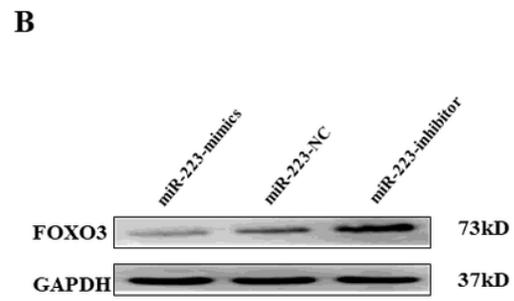
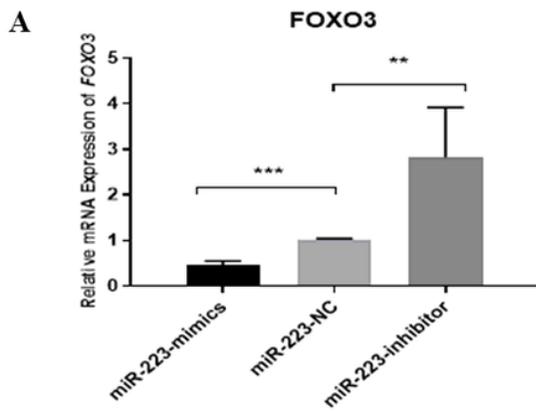


Figure 3

Gga-miR-223 negatively regulates FOXO3 expression.

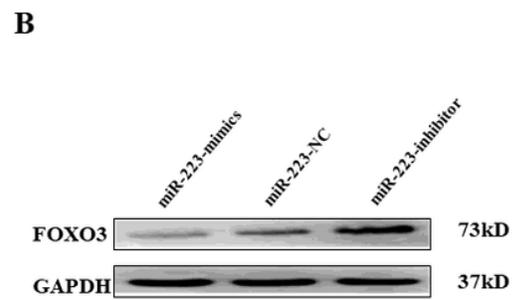
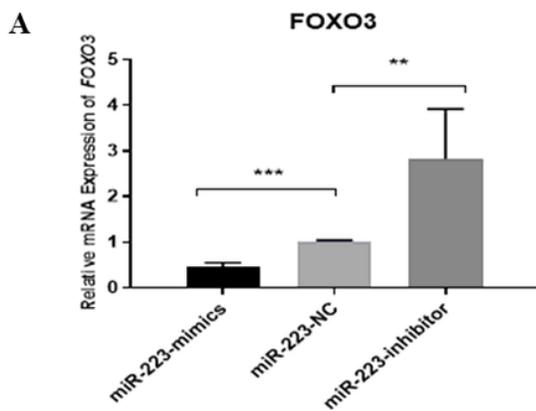


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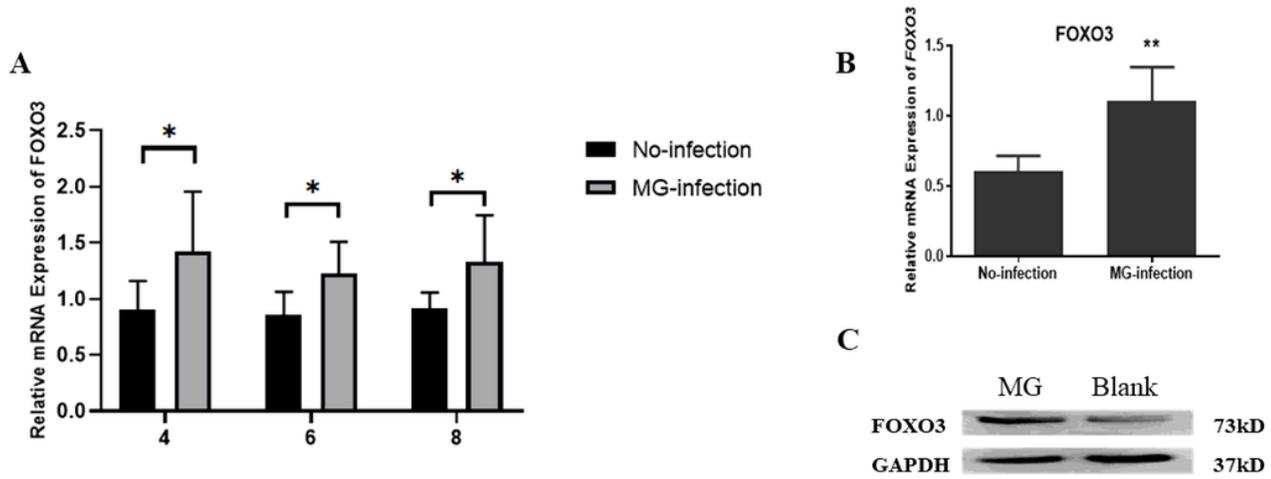


Figure 4

MG-infection up-regulate FOXO3 expression.

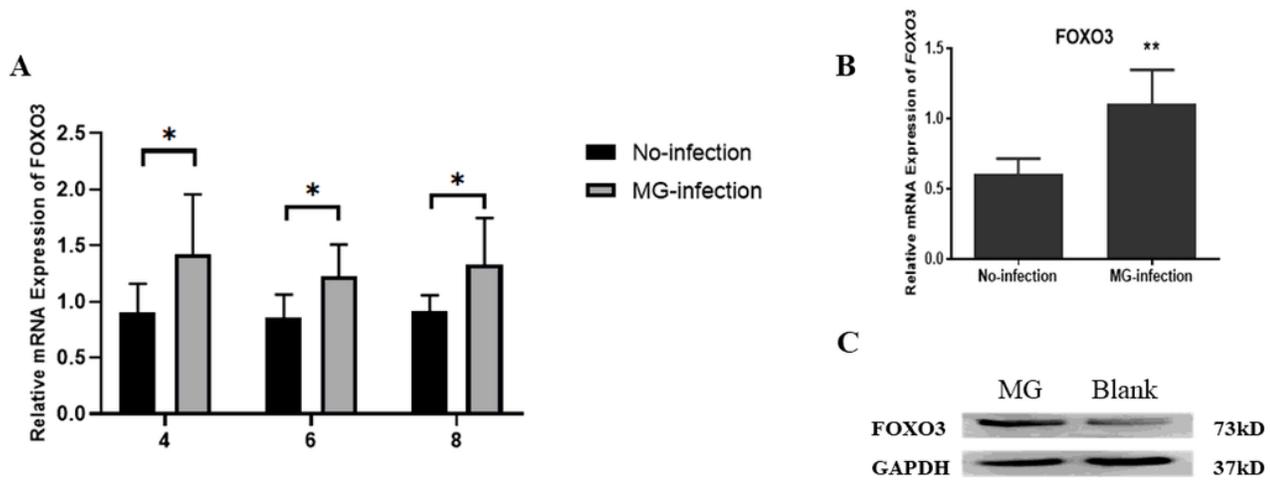


Figure 4

MG-infection up-regulate FOXO3 expression.

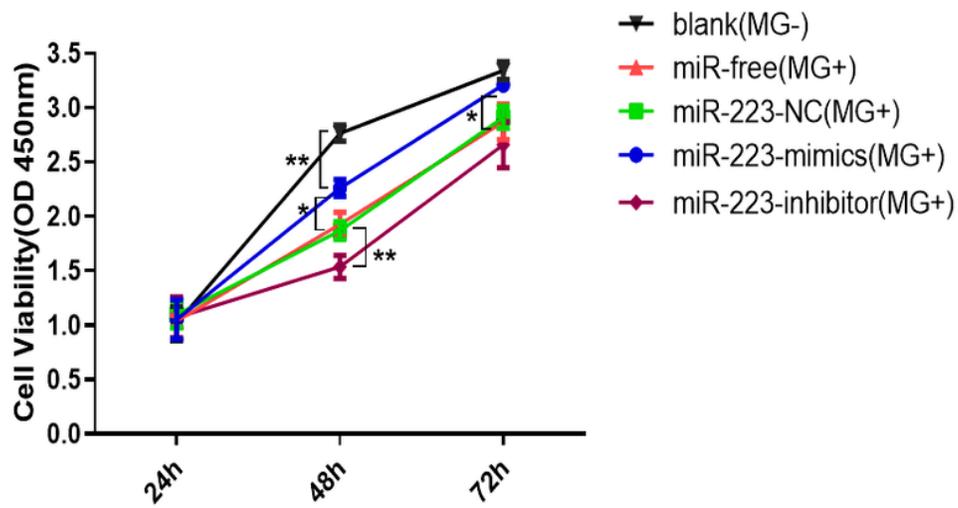


Figure 5

Over-expression of gga-miR-223 promotes DF-1 cell proliferation.

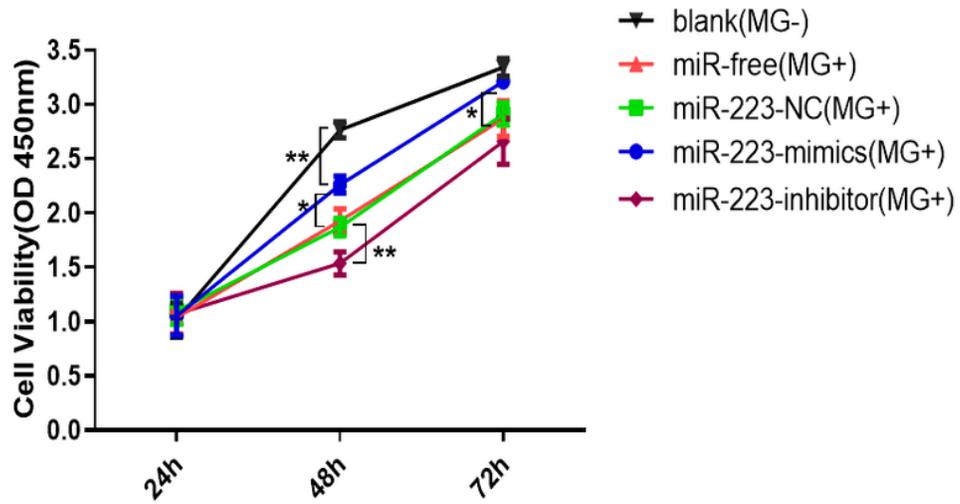


Figure 5

Over-expression of gga-miR-223 promotes DF-1 cell proliferation.

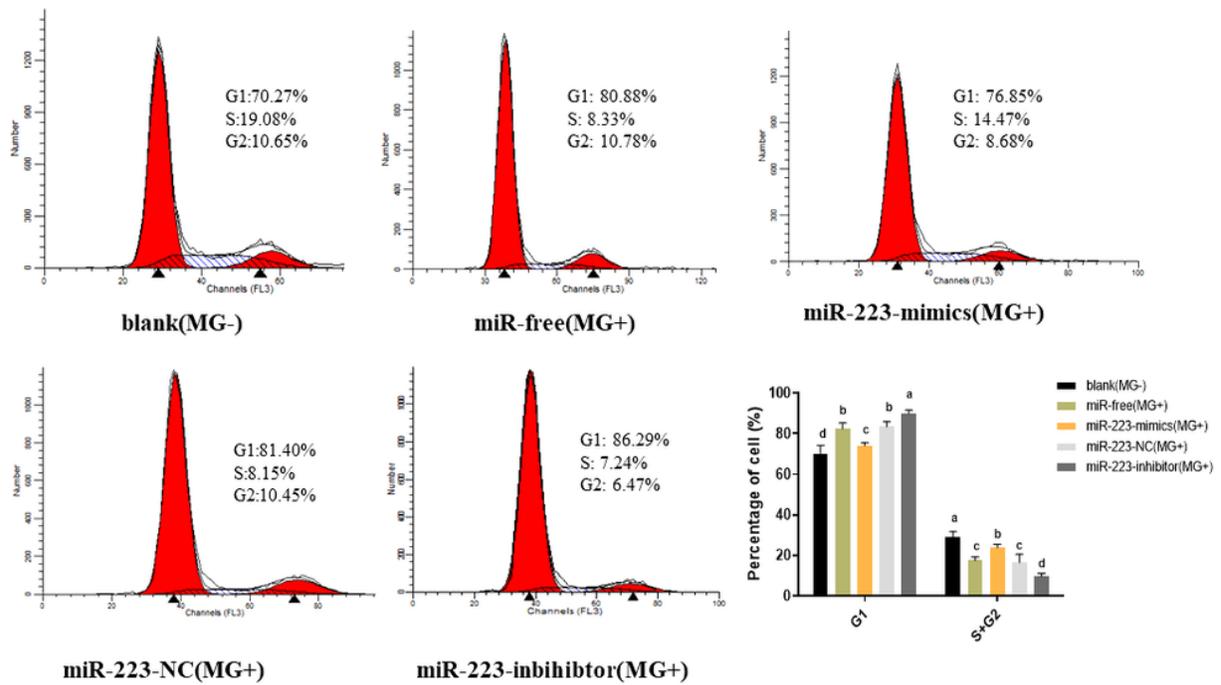


Figure 6

Overexpression of gga-miR-223 transformed DF-1 cells from G1-phase to S- and G2-phase.

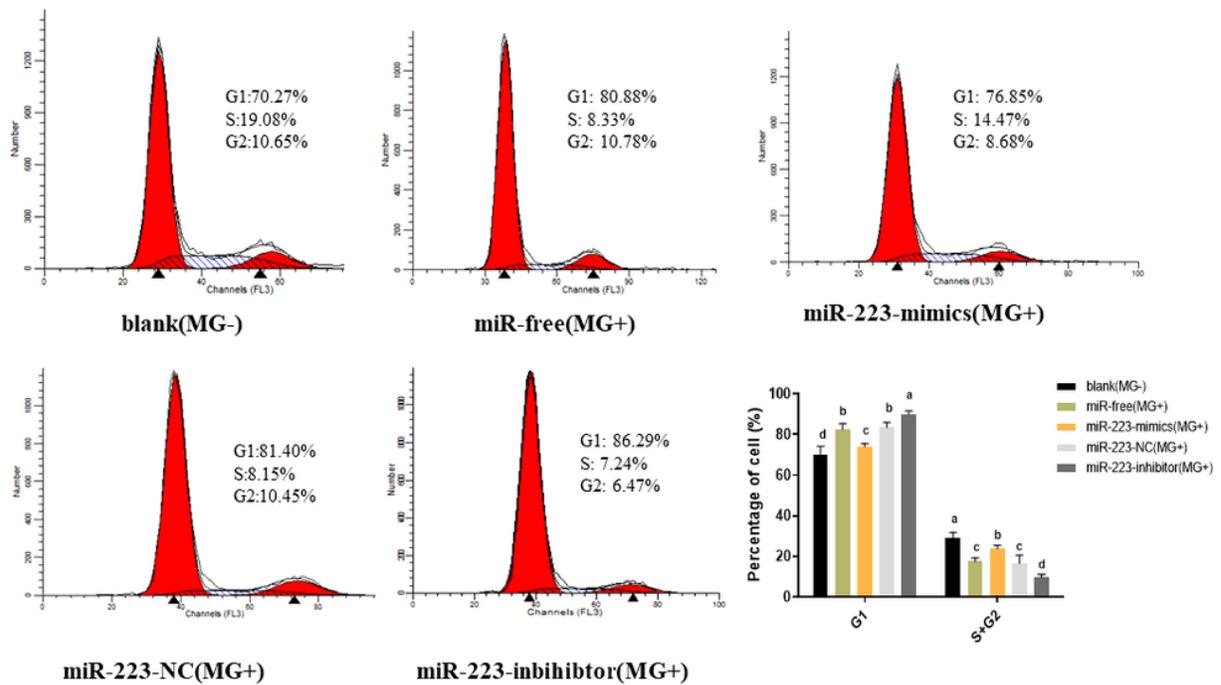


Figure 6

Overexpression of gga-miR-223 transformed DF-1 cells from G1-phase to S- and G2-phase.

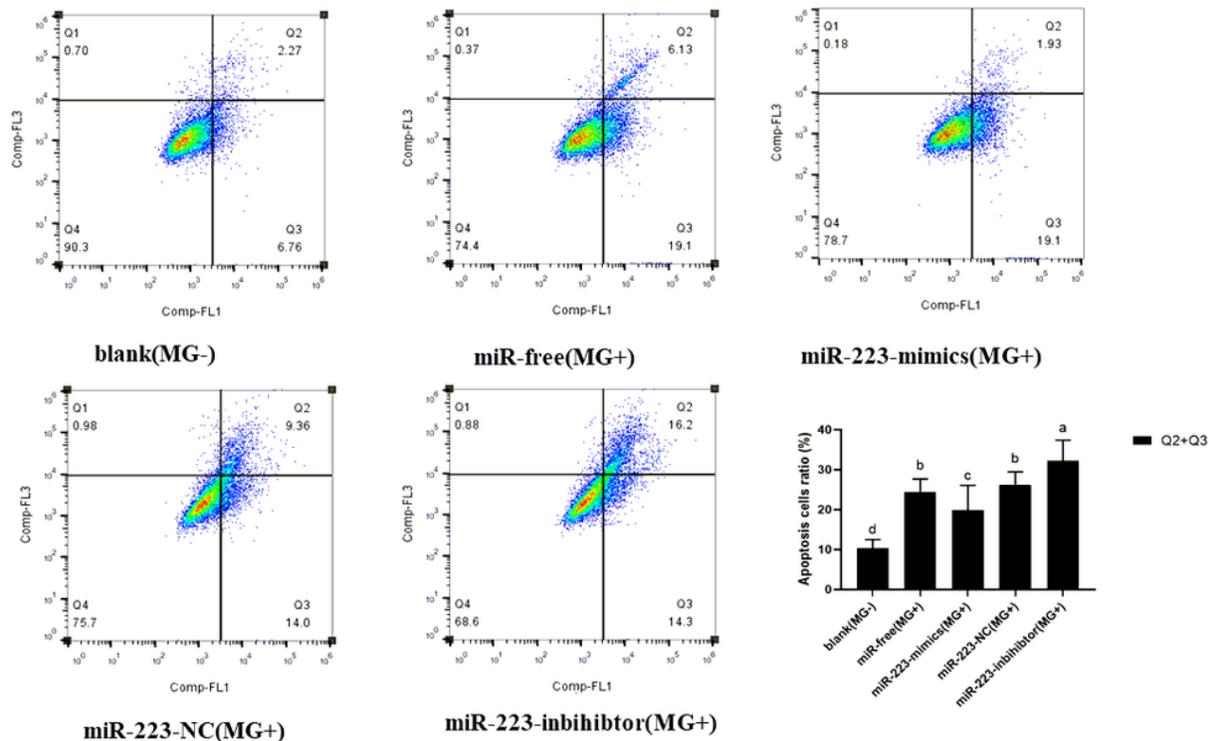


Figure 7

Effects of overexpression and inhibition of gga-miR-223 on the cell apoptosis of DF-1 cells with MG infected.

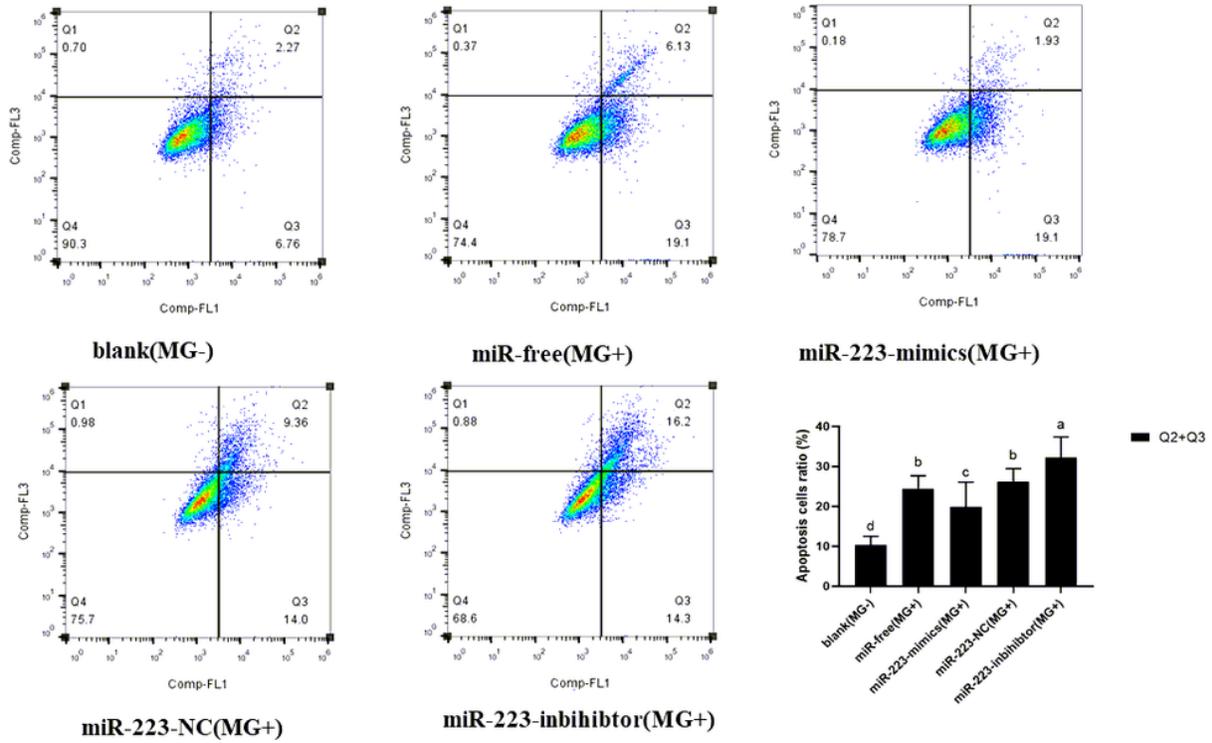


Figure 7

Effects of overexpression and inhibition of gga-miR-223 on the cell apoptosis of DF-1 cells with MG infected.

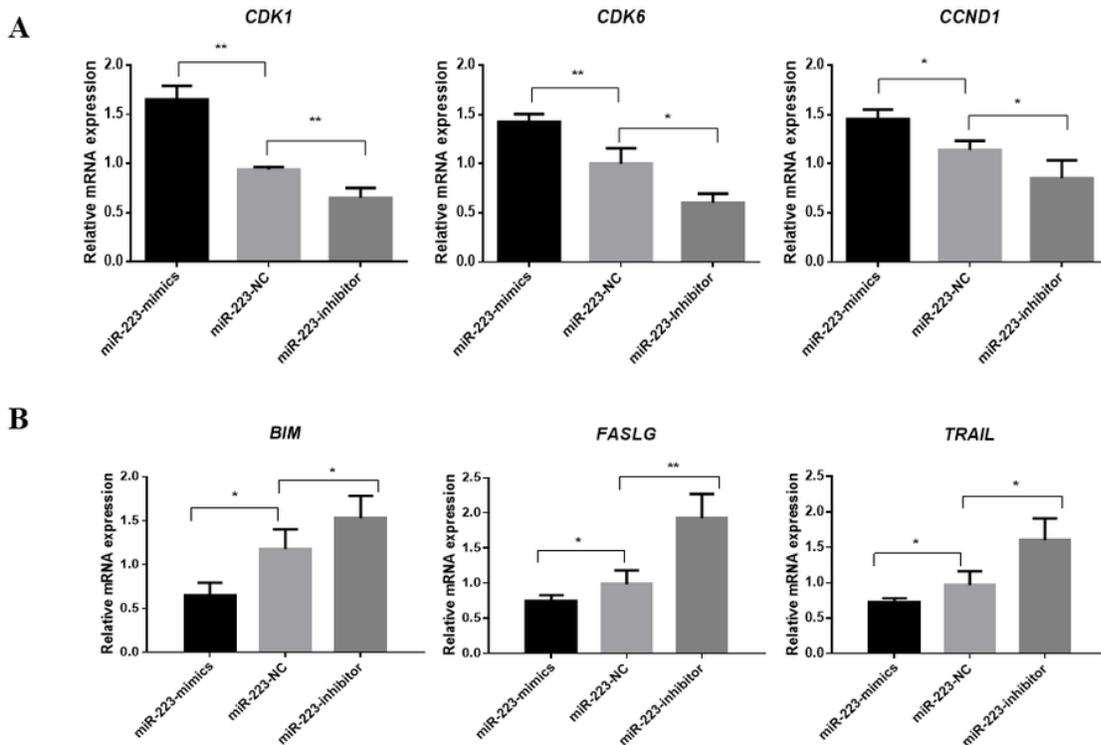


Figure 8

Effects of gga-miR-223 on the cycle and apoptosis genes in DF-1 cells.

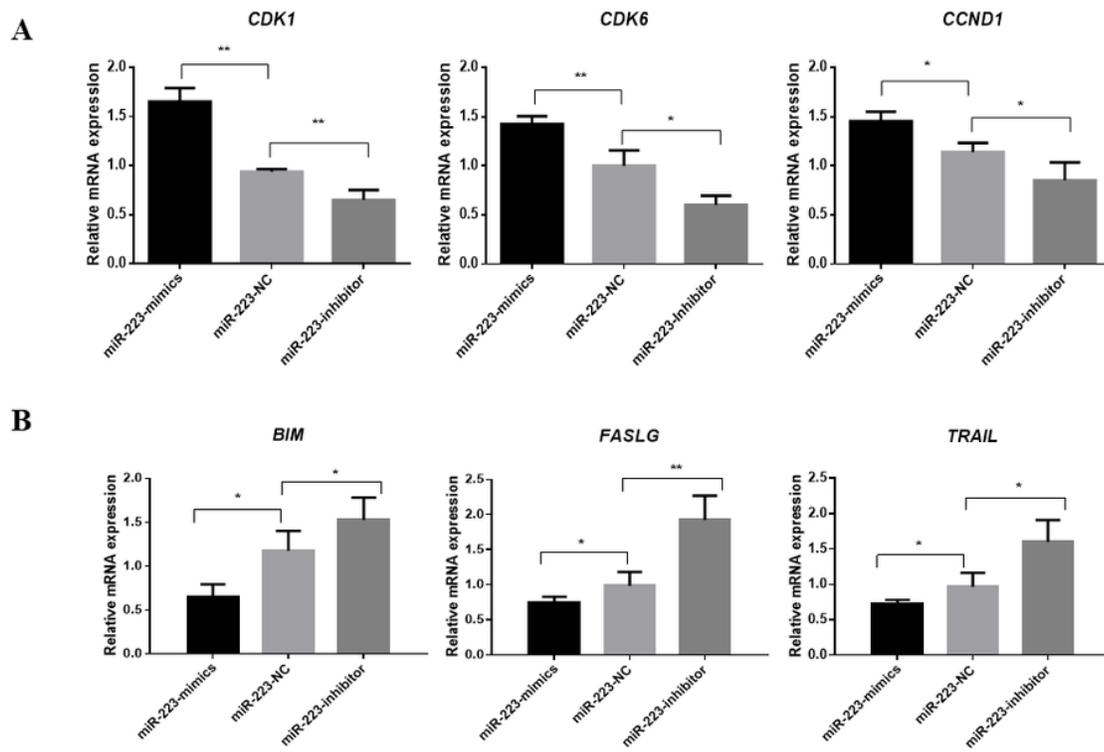


Figure 8

Effects of gga-miR-223 on the cycle and apoptosis genes in DF-1 cells.

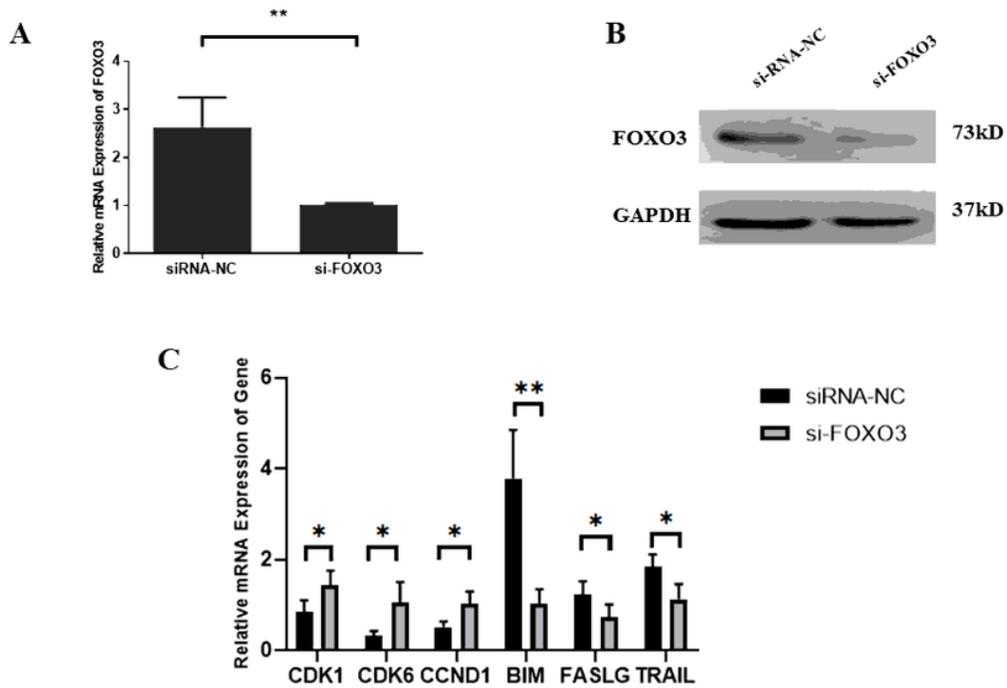


Figure 9

Effect of knock-down of FOXO3 in DF-1 on cell proliferation.

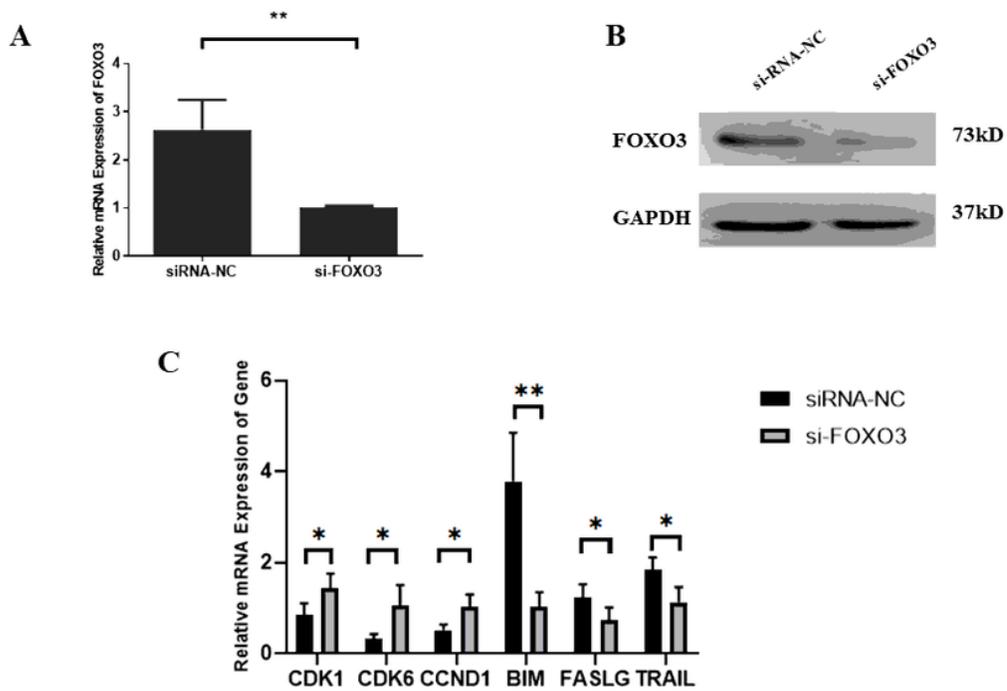


Figure 9

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