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ORIGINAL ARTICLE

MicroRNA-322 regulates the growth, chemosensitivity, migration and invasion of breast cancer cells by targeting NF-kB1

Junbo Hu[1], Peng Guo[1], Yanli Zhang[1], Ziming Huang[2], Bo Chen[2]

[1]Department of Pathology, Maternal and Child Health Hospital of Hubei Province, Hubei Province, China, 430070; [2]Department of Mammary Surgery, Maternal and Child Health Hospital of Hubei Province, Hubei Province, China, 430070

Summary

Purpose: *Breast cancer is one of the leading causes of mortality in women across the globe. Herein, the role and therapeutic implications of miR-322 were investigated in breast cancer.*

Methods: *An array of breast cancer cell lines and a normal cell line were used in this study. The expression of miR-322 was determined by quantitative realtime polymerase chain reaction (qRT-PCR). Lipofectamine 2000 reagent was used to perform transfections and MTT assay was used to determine the cell viability. DAPI and annexin V/propidium iodide (PI) assays were used to detect apoptosis. Wound healing and transwell assays were used to monitor cell migration and invasion, respectively. Protein expression was determined by western blot analysis.*

Results: *The expression of miR-322 was found to be remarkably suppressed in breast cancer cells. Overexpression of miR-322 led to considerable decline in the proliferation rate and colony formation of the MCF7 breast cancer cells due to induction of apoptosis. The overexpression of miR-322 caused a significant increase in Bax and decrease in Bcl-2 expression and also enhanced the sensitivity of MCF7 cells to cisplatin and decreased their migration and invasive potential. The TargetScan analysis showed NF-kB1 to be the target of miR-322. Additionally, NF-kB1 was remarkably upregulated in all the breast cancer cells. However, miR-322 overexpression resulted in depletion of NF-kB1 expression in MCF7 cells. Silencing of NF-kB1 also decreased the proliferation rate and colony formation of the MCF7 cells.*

Conclusion: *To conclude, miR-322 may exhibit therapeutic implications in breast cancer treatment and warrants further investigation.*

Key words: *breast cancer, microRNA, apoptosis, NF-kB1, invasion*

Introduction

Accounting for 14% of all the cancer-related deaths, breast cancer is one of the destructive cancers throughout the world. Annually, 0.4 million deaths are caused by breast cancer alone [1]. Despite advancements made in the field of breast cancer research, the survival of advanced-stage breast cancer is still very poor. Additionaly, late diagnosis of breast cancer hurdles the efficient treatment of ovarian cancer [2]. Moreover, the efficiency of the currently available chemotherapy is poor and the side effects could be severe. Finally, the development of drug resistance among breast cancer cells further complicates the problem [3]. Therefore, the detection of novel therapeutic targets or development of highly effective and safer therapeutic agents may prove beneficial in the breast cancer treatment [4]. Recent studies have shown that microRNAs (miRs) may exhibit therapeutic implications in treating a diversity of human diseases such as cancer [5]. MiRs control the expression of target

Corresponding author: Bo Chen, PhD. Department of Mammary Surgery, Maternal and Child Health Hospital of Hubei Province, No.745 Wuluo Rd, Wuhan City, Hubei province, China, 430070.
Tel/Fax: +86 027 87161012; Email: BrendenWoodrcb@yahoo.com
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genes via post transcriptional regulation and are around 19-23 nucleotides in length [6]. MiR-322 has been shown to control a diverse array of molecular processes [7]. It has been reported to regulate the expression of inflammatory cytokines and growth of macrophages [8]. MiR-322 has also been shown to regulate phosphorylation of tau protein [9]. In yet another study, miR-322 has also been reported to play a role in gut epithelial homeostasis [10]. Similarly, miR-322 has been found to control the differentiation of muscle cells [11].

However, there is no report on the role and therapeutic implicates of miR-322 in any type of cancer. Against this background, this study was undertaken to ascertain the role and therapeutic implications of miR-322 in breast cancer.

Methods

Cell lines

Breast cancer cell lines EMT6, BT-20, MCF7, SK-BR-3 and normal breast cell line (Hs 841.T) were procured from American Type Culture Collection. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) having 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum (FBS).

RNA isolation and qRT-PCR

RNA was extracted from the breast cancer cells by using TRIzol reagent (Invitrogen, Carlsbad, California, USA). RNA was then purified with RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was then synthesized with miScript Reverse Transcription Kit (Qiagen). Thereafter, cDNA was amplified using SYBR Premix Ex *Taq*™ (TaKaRa, Otsu, Shiga, Japan).

Cell transfection

The miR-322 mimics and miR-negative control (NC) were synthesized by RiboBio (Guangzhou, China). Transfection was then carried out by the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. As the MCF7 cells reached 80% confluence, the appropriate concentrations of miR-322 mimics or NC were transfected into these cells.

MTT cell viability

Because MCF7 cell line showed the lowest expression of miR-322 among all cell lines, only this cell line was used for further studies. The MTT cell viability assay was employed for the monitoring of the MCF7 cell proliferation at 0, 12, 24, 48 and 96 h time periods. In brief, the MCF7 cells were cultured for 24h after transfection with appropriate miR constructs. Then, 500 µg/ml MTT solution was added to the cell culture for 4 h. The formazan crystals which were formed from MTT were dissolved with DMSO (10%). To monitor the cell viability, the absorbance was taken at 570 nm with a spectrophotometer.

DAPI and annexin V/PI assay

The MCF7 cells (0.6×10^6) were cultured in 6-well plates for 24 h at 37°C. Subsequently, 25 µl of cell culture were put onto glass slides and stained with DAPI. The slides were then cover-slipped and examined under fluorescence microscope. ApoScan kit was used to determine the apoptotic MCF7 cells. To determine the percentage of apoptosis, the transfected MCF7 cells (5×10^5 cells per well) were incubated at 37°C for 24 h. This was followed by the staining of these cells with annexin V-FITC/propidium iodide (PI). The percentage of apoptotic MCF7 cells was then determined by flow cytometry.

Target identification

The miR-322 target was identified by TargetScan online software (http://www.targetscan.org) using default parameters.

Dual-luciferase reporter assay

The miR-322 mimics or miR-NC were co-transfected with Plasmid pGL3-NF-kB1´-UTR-WT or pGL3-NF-kB1´-UTR-MUT into MCF7 cells. Dual-luciferase reporter assay (Promega) was carried out at 48 h after transfection. *Renilla* luciferase used for normalization.

Wound healing assay

After 24 h of miR-322 mimics and miR-NC transfection into MCF7 cells, the Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Mass, USA) was removed and cells were subjected to phosphate-buffered saline (PBS) washing. A sterile pipette tip was employed to scratch a wound in each well and cells were washed again and a picture was taken. The plates were subjected to culturing at 24 h and a picture was taken again under an inverted microscope (Leica, Germany).

Transwell assay

Transwell assay was used to assess the effects of miR-322 on the invasion of MCF7 cells. In brief, the MCF7 cells were transfected with miR-322 mimics and NC and around 200 ml cell culture were placed onto the upper chambers and only medium was placed in the chambers. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded to the lower chamber were fixed with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200x magnification.

Western blotting

The transfected MCF7 cells were harvested with centrifugation and were then lysed in lysis buffer containing the protease inhibitor. Around 45 µg of proteins from each sample were then separated on 10% SBS-PAGE and followed by transferring to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Thereafter, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the

membranes were incubated with secondary antibodies. Finally, the protein signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalisation.

Statistics

All the experimental procedures were performed in triplicate. The values obtained are presented as mean of these three experiments ± SD. P<0.05 was considered statistically significant. The statistical analysis was performed by Student's t-test using GraphPad prism 7 software.

Results

The miR-322 is downregulated in breast cancer cells

The qRT-PCR was employed to estimate the expression of miR-322 in normal and breast cancer cells. The results revealed that miR-322 was significantly downregulated in the breast cancer cells (Figure 1A). The expression of miR-322 was found to be up to >5-fold lower in the breast cancer cells compared to normal cells. The MCF7 cell line showed the lowest expression of miR-322 across all the cell lines.

miR-322 inhibits the proliferation and colony formation of MCF7 cells

Next, the effects of miR-322 overexpression were assessed on the proliferation rate of the MCF7 breast cancer cells. For this, the miR-322 mimics or miR-NC were transfected into the MCF7 cells. The overexpression of miR-322 was confirmed by qRT-PCR which showed 6.05-fold increase in miR-322 expression (Figure 1B). The transfected MCF7 cells were then subjected to MTT assay and the proliferation rate was monitored at different time periods.

The results showed that miR-322 overexpression led to a remarkable reduction in the proliferation rate of the MCF7 cells (Figure 1C). Additionally, the colony formation assay showed that miR-322 overexpression resulted in the suppression of the colony formation of the MCF7 cells (Figure 1D).

miR-322 induces apoptosis in MCF7 cells

DAPI staining assay was performed to decipher if miR-322 inhibits the proliferation rate of the MCF7 cells via induction of apoptosis. The results of DAPI staining unequivocally showed that

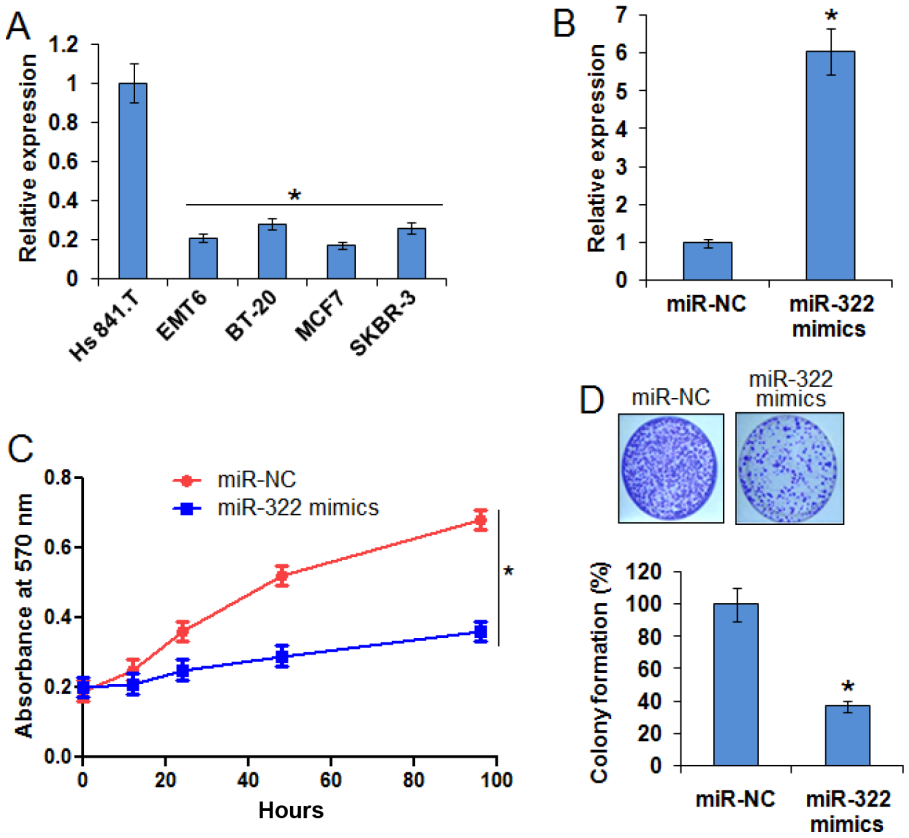

Figure 1. A: Relative expression of miR-322 in normal Hs841.T and breast cancer cells. **B:** Expression of miR-322 in miR-NC and miR-322 mimics transfected MCF7 cells. **C:** Cell viability of miR-NC and miR-322 mimics transfected MCF7 breast cancer cells. **D:** Colony formation of miR-NC and miR-322 mimics transfected MCF7 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p< 0.05).

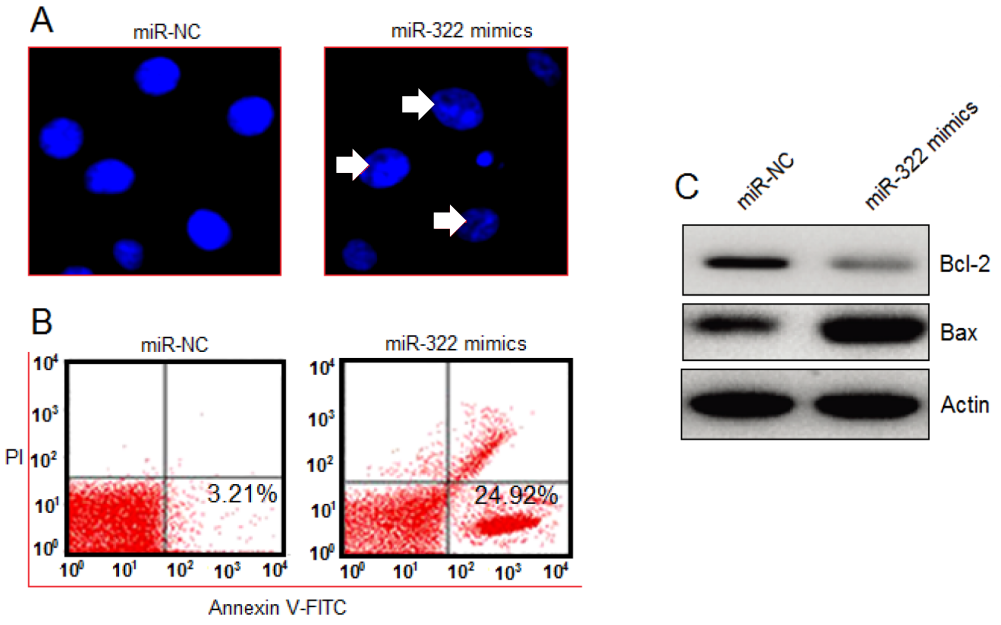

Figure 2. A: DAPI staining and **B:** annexin V/PI staining showing apoptosis in miR-NC and miR-322 mimics transfected MCF7 breast cancer cells. **C:** Western blot analysis showing the expression of Bcl-2 and Bax in miR-NC and miR-322 mimics transfected MCF7 breast cancer cells (arrows show apoptotic cells). The experiments were performed in triplicate.

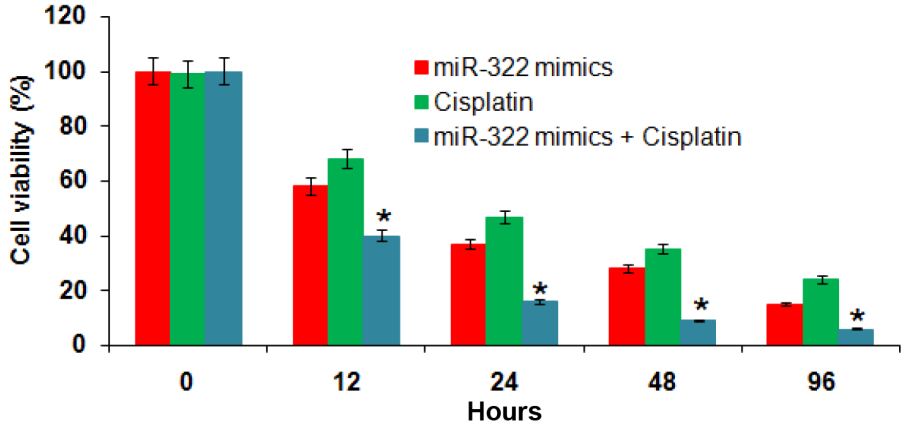

Figure 3. Effect of miR-322 on the cisplatin sensitivity of MCF7 breast cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

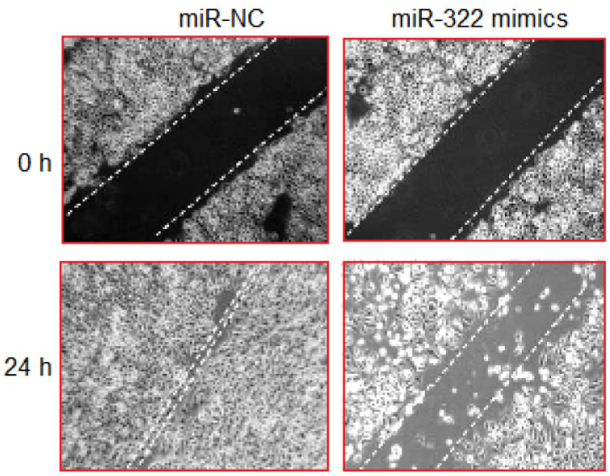

Figure 4. Wound healing assay showing migration of miR-NC and miR-322 mimics transfected MCF7 breast cancer cells. The experiments were performed in triplicate.

miR-322 overexpression resulted in apoptosis of the MCF7 cells (Figure 2A). Annexin V/PI staining showed that the percentage of the apoptosis in miR-322 mimics-transfected MCF7 cells was 24.92% as compared to 3.21% in NC-transfected cells (Figure 2B). The overexpression of miR-322 also resulted in increase of Bax expression and decrease of Bcl-2 expression in MCF7 breast cancer cells (Figure 2C).

miR-322 enhances the chemosensitivity of MCF-7 cells

To unveil if miR-322 had any effect on the cisplatin sensitivity of the MCF7 cells, the cells were subjected to treatment with 1.5 μM cisplatin or miR-322 mimics transfection or cisplatin treatment plus miR-322 mimics transfection. The proliferation rate of all these groups of cells was

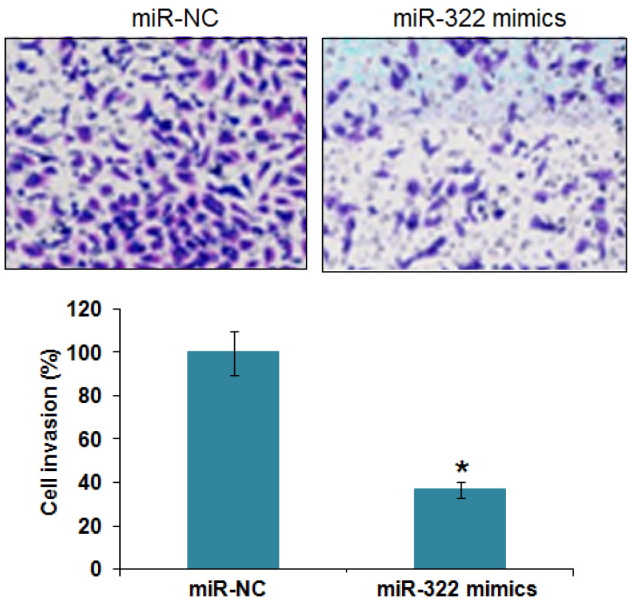

Figure 5. Transwell assay showing invasion of miR-NC and miR-322 mimics transfected MCF7 breast cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

monitored by MTT assay at 0, 12, 24, 48, and 96 h time periods. The results showed that the proliferation rate of miR-322 mimics or cisplatin-treated cells was significantly higher as compared to MCF7 cells treated cisplatin plus transfected with miR-322 mimics (Figure 3).

miR-322 suppresses migration and invasion of MCF7 cells

The effect of miR-322 on the migration of the MCF7 breast cancer cells was examined by wound healing assay. It was found that miR-322 inhibited the migration of MCF7 cells as evidenced from the wound width (Figure 4). On the other hand, transwell assay showed that miR-322 significantly suppressed the invasion of the MCF7 cells. The invasion of the MCF7 cells was suppressed by up to 62.5% upon miR-322 overexpression (Figure 5).

miR-322 targets NF-kB1 in MCF7 breast cancer cells

The TargetScan analysis showed that miR-322

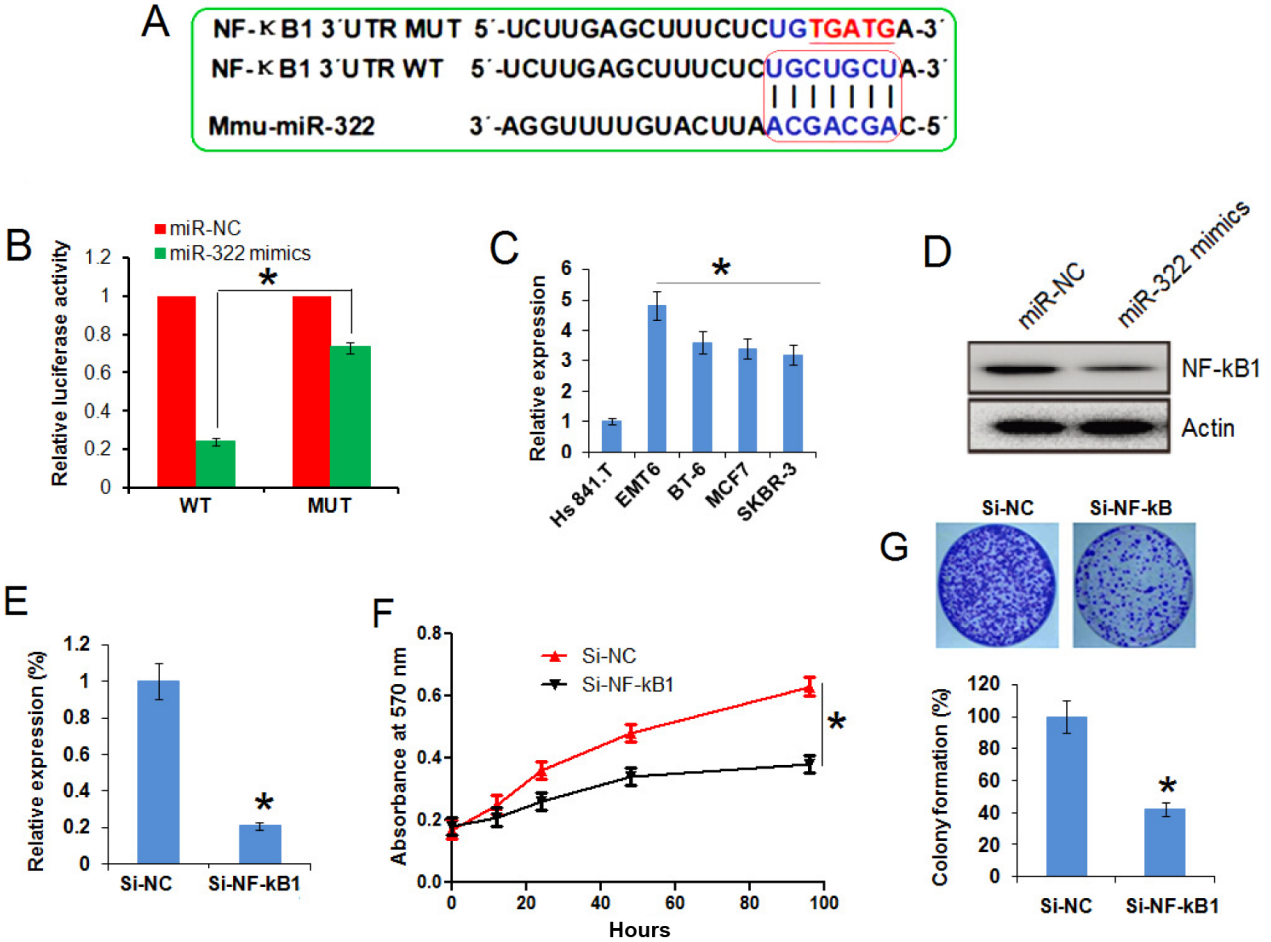

Figure 6. A: TargetScan analysis showing NF-kB1 as the target of miR-322. **B:** Dual luciferase assay. **C:** expression of NF-kB1 in normal and breast cancer cell lines. **D:** Expression of NF-kB1 in miR-NC or miR-322 mimics transfected MCF7 cells as depicted by western blot analysis. **E:** Expression of NF-kB1 in si-NC or Si-NF-kB1 transfected MCF7 cells. **F:** Cell viability of the si-NC and si-NF-kB1 transfected MCF7 breast cancer cells. **G:** Colony formation of the si-NC and si-NF-kB1 transfected MCF7 breast cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

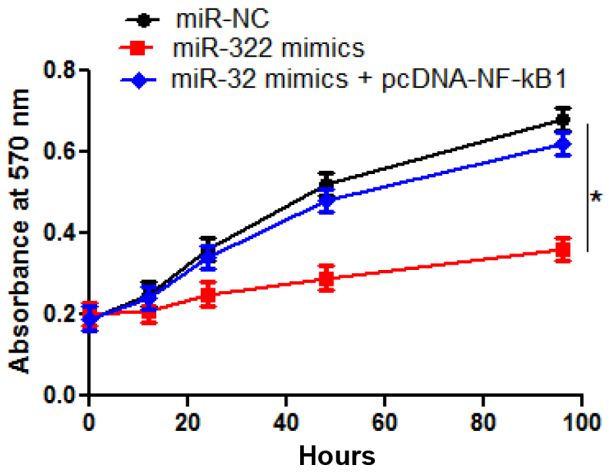

Figure 7. Rescue assay showing the effect of NF-kB1 overexpression on the growth inhibition of miR-322 overexpression in MCF7 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

targets NF-kB1 in MCF7 breast cancer cells (Figure 6A). Dual luciferase also confirmed NF-kB1 as the target of miR-322 (Figure 6B). Hence, the expression of NF-kB1 was examined in all the breast cancer cells and it was found that the expression of NF-kB1 was considerably upregulated in all the breast cancer cells (Figure 6C). However, miR-322 overexpression caused depletion of NF-kB1 protein levels in MCF7 cells (Figure 6D). To assess the impact of NF-kB1 on MCF7 cell proliferation, NF-kB1 was silenced in MCF7 cells (Figure 6E) and it was found that NF-kB1 silencing caused decrease in the proliferation rate and colony formation of the MCF7 cells (Figure 6F and 6G). However, rescue assays showed that restoration of NF-kB1 expression in MCF7 cells overexpressing miR-322 could rescue the inhibitory effects of miR-322 overexpression on cell proliferation (Figure 7).

Discussion

Breast carcinoma is one of the prevalent types of malignancies in women across the globe [12]. Late diagnosis, lack of effective and safer drugs and lack of novel therapeutic targets are the main obstacles that limit its treatment [2,3]. Because of their diverse roles in cancer-related processes miRs are believed to have the potential to act as exceptional therapeutic targets [13]. In this study, the therapeutic implications of miR-322 were explored for the first time in breast cancer and the results showed that the expression of miR-322 is downregulated in breast cancer cells, ie., the expression of miR-322 was considerably lower in breast cancer cells than the normal cells. This is in agreement with the previous findings wherein miRs have been shown to be dysregulated in breast cancer cells. For example, miR-224 is upregulated in breast cancer [14] while as miR-99a-5p is downregulated in breast cancer cells [15]. In this study we found that overexpression of miR-322 in the MCF-7 breast cancer cells resulted in suppression of proliferation and the colony formation. Consistently, previous studies have also shown that some miRs inhibit the proliferation of breast cancer cells [16,17]. The miR-132 targets FOX1 to inhibit the proliferation of breast cancer cells [16]. Similarly, miR-101 7 targets HMGB1 to suppress the growth of breast cancer cells [17]. To unveil the molecular mechanisms, we performed the DAPI assay of the miR-322 mimics-transfected breast cancer cells to ascertain if miR-322 overexpression induces apoptosis. Interestingly, it was found that overexpression of miR-322 promotes the apoptotic cell death of the MCF7 breast cancer cells. Moreover, miR-322-promoted apoptosis in the MCF7 breast cancer cells was also accompanied with increase in Bax and decrease of the Bcl-2. Several of miRs have been shown to regulate the chemosensitivity of cancer cells, for example miR-181c increases the chemosensitivity of breast cancer cells [18]. Consistently, in the present study miR-322 was found to enhance the chemosensitivity of MCF7 cells to cisplatin. To ascertain the impact of miR-322 on the migration and invasion of the MCF7 breast cancer cells, wound healing and transwell assay were performed and it was found that miR-322 overexpression suppresses the invasion of the MCF7 cells.

MiRs have been shown to perform their functions by modulating the expression of their target genes [16]. Herein, we found that miR-322 targets NF-kB1 in breast cancer cells and its overexpression suppresses the NF-kB1 expression. This was also supported by a previous study wherein miR-322 has been shown to target NF-kB1 [8]. All these findings indicate the potential therapeutic implications of miR-322 in breast cancer treatment.

Conclusion

The findings of the present study revealed that miR-322 is overexpressed in breast cancer cells and inhibits the proliferation of the breast cancer cells by targeting NF-kB1. Additionally, miR-322 also enhances the chemosensitivity to cisplatin and suppresses the migration and invasion of the breast cancer cells. Therefore, miR-322 may exhibit therapeutic implications in breast cancer treatment.

Conflict of interests

The authors declare no conflict of interests.

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