

RESEARCH ARTICLE

Effects of miR-155 Antisense Oligonucleotide on Breast Carcinoma Cell Line MDA-MB-157 and Implanted Tumors

Shu-Rong Zheng, Gui-Long Guo*, Qi Zhai, Zhang-Yong Zou, Wei Zhang

Abstract

Diverse studies have shown that miR-155 is overexpressed in different tumor types. However, the precise molecular mechanism of the ectopic expression of miR-155 in breast cancer is still poorly understood. To further explore the role of miR-155 in breast tumorigenesis, we here assessed the influence of miR-155 antisense oligonucleotide (miR-155 ASO) on MDA-MB-157 cell viability and apoptosis *in vitro*. Furthermore, the effects of inhibitory effects of miR-155 on the growth of xenograft tumors *in vivo* were determined with performance of immunohistochemistry to detect expression of caspase-3, a pivotal apoptosis regulatory factor, in xenografts. Transfection efficiency detected by laser confocal microscope was higher than 80%. The level of miR-155 expression was significantly decreased ($P < 0.05$) in the cells transfected with miR-155 ASO, compared with that in cells transfected with a negative control. After being transfected with miR-155 ASO, the viability of MDA-MB-157 cells was reduced greatly ($P < 0.05$) and the number of apoptotic cells was increased significantly. Additionally, miR-155 ASO inhibited the growth of transplanted tumor *in vivo* and significantly increased the expression of caspase-3. Taken together, our study revealed that miR-155 ASO can induce cell apoptosis and inhibit cell proliferation *in vitro*. Moreover, miR-155 ASO could significantly repress tumor growth *in vivo*, presumably by inducing apoptosis via caspase-3 up-regulation. These findings provide experimental evidence for using miR-155 as a therapeutic target of breast carcinoma.

Keywords: Breast neoplasms - microRNA-155 - antisense oligonucleotide - MDA-MB-157 cells - nude mice

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Introduction

Breast cancer is by far the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008 (Jemal et al., 2011). Despite previous research and resources dedicated to elucidating the mechanisms of breast cancer, the precise molecular mechanisms of its initiation and progression remain poorly understood.

MiRNAs are small (20-24 nucleotides [nt]) noncoding RNA gene products that have become known as important regulators of various cellular processes by post-transcriptionally modulate gene expression (Ambros, 2003; Bartel., 2009). There are now over 600 miRNAs estimated to play roles in humans (Griffiths-Jones., 2006), and about 30% of all genes are regulated by miRNAs (Yu, 2006). MiRNAs are key regulators of cellular differentiation (Lee et al., 1993; Chen et al., 2004), proliferation (Hayashita et al., 2005; He et al., 2005), cell survival and apoptosis (Ambros., 2003; Brennecke et

al., 2003), and have been reported to be associated with tumorigenesis acting as oncogenes (He et al., 2005; Tam and Dahlberg., 2006; Papagiannakopoulos et al., 2008) or suppressors (Hammond., 2007; Dong and Lou., 2012). In particular, miR-155 has emerged as a key onco-mir, since it is one of the most consistently up-regulated miRNAs in a wide range of cancers (Yin et al., 2010; Yip et al., 2011; Donnem et al., 2012; Zheng et al., 2012).

Although miR-155 has been found to be up-regulated in breast cancer, its role in breast tumorigenesis has not yet been clarified. Therefore we chose MDA-MB-157 as a breast carcinoma cell line highly expressing miR-155 (Kong et al., 2010), as our primary experimental material. In this study, we first aimed to confirm that the effects of synthesized miR-155 ASO on MDA-MB-157 cells growth and proliferation *in vitro*. Next, we evaluated the role of miR-155 ASO in tumor formation in immunocompromised mice inoculated sc with MDA-MB-157 cells. Finally, we detected the expression of caspase-3 in tumor xenografts by immunochemistry to further explore the effects and the precise molecular mechanisms of the intervening measure targeting miR-155 tumorigenesis *in vivo*.

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Materials and Methods

Design and synthesis of miR-155 ASO sequences

The mature miRNA sequences are available from the miRNA Registry. The sequences of miRNA ASO were designed, according to the principle of sequences complementary to the mature mRNA. The ASO and the scrambled negative control (SCR) sequences used in this study are listed in Table 1. Both of them were chemically synthesized and 2'-OMe modified by Shanghai GenePharma Co., Ltd (Shanghai, China) and stored at -20°C.

Cell lines and transfection

Breast cancer cell line (MDA-MB-157) was obtained from ATCC and grown according to ATCC recommended culture conditions. Twenty four hours before transfection, MDA-MB-157 cells in the exponential phase of growth were seeded in 96- or 6-well plates (Costar) and allowed to grow overnight. The cells were then transfected with oligonucleotides using LipofectamineTM2000 reagent (Invitrogen) in OPTI MEMI for 6 hours. Transfection complexes were prepared according to the manufacturer's instructions. At the end of transfection, the cells were incubated in medium containing 10% fetal calf serum (FCS). Transfection efficiency was detected by laser confocal microscope.

Real-time PCR for quantitative analysis of miR-155

MDA-MB-157 cells were incubated in 6-well plates and transfected with 75nM oligonucleotides using the LipofectamineTM2000 reagent for 48 hours. The same process was followed as described above. Briefly, miRNAs were isolated by miRcute miRNA isolation kit (Tiangen, China). The extracted products were then done reverse transcription (RT) reaction using miRcute miRNA first-strand cDNA synthesis kit (Tiangen, China). The synthesized first-strand cDNA was kept at -20°C. All procedures were performed according to the instructions provided by the manufacturer. The miR-155 level was quantified by quantitative reverse transcription-PCR (qRT-PCR) with 5s small nuclear RNA as an internal normalized reference. The qPCR was performed on ABI 7500 Real-Time PCR System (ABI, USA) with miRcute miRNA qPCR detection kit (Tiangen, China), which included specific reverse primer for miRNAs. The qPCR reaction system contained 10ul 2×miRcute miRNA premix, 0.4ul miR-155 primer, 0.4ul reverse primer, 2ul ten fold serial dilution of miRNA first-strand cDNA and 7.2ul RNase-free water. The procedure for PCR was 94°C 2 min; 94°C 20s, 60°C 30s, 72°C 30 s, 45 cycles. The sequences of the forward primers are listed in Table 1.

Table 1. The Sequences of Oligonucleotide and Primers for the Analysis of miR-155 Expression

Gene name	Sequence (5'→3')
miR-155 ASO	ACCCCUAUCAAGAUUAGCAUUA
5'FAM SCR	CAGUACUUUGUGUAGUACAA
miR-155-F	GGTTAATGCTAATCGTGATAGGGGTAA
5S rRNA-F	GTCTACGGCCATACCACCCTGAAC

(F) Forward primer

Cell Viability and Apoptosis Assays

The effect of miR-155 ASO on MDA-MB-157 cells viability was determined by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt (WST-8) assay kit (CKK-8, Dojindo, Kumamoto, Japan). Twenty four hours before transfection, 1×10⁴ MDA-MB-157 cells were seeded per well in 96-well plates and allowed to grow overnight. The cells were then transfected with three different concentration of miR-155 ASO (25 nM, 50 nM and 75 nM, respectively) and highest concentration of SCR siRNA (75 nM) using lipofectamineTM2000 according to the manufacture's protocol. After 48h, WST-8 was added into each well for 1 hour before the measurement according to the manufacturer's instructions. The absorbance at 450 nm was measured by a microplate reader. Inhibition rate = (1-absorbance of treated cells/control cells)×100% (Zhang et al., 2002). Apoptosis assay was detected with Annexin V-FITC/PI Apoptosis Detection Kit (Roche). MDA-MB-157 cells were transfected with miR-155 ASO or SCR as previously described for 6 hours, and incubated in medium containing 10% FCS for another 48 hours in 6-well plates. Cells were collected and double stained with FITC conjugated annexin V and propidium iodide (PI). For each sample, data from approximately 1×10⁴ cells were recorded in the list mode on logarithmic scales. Apoptosis and necrosis were analysed by quadrant statistics on PI-negative, annexin V-positive cells and both positive cells, respectively.

Xenograft assays in nude mice

To evaluate in vivo tumorigenesis, breast carcinoma xenografting mouse model was used. 4-week-old male BALB/c athymic nude mice were obtained from the SLAC Laboratory Animals Co Ltd (Shanghai, China) and prepared for tumor implantation. All experimental procedures involving animals were performed in accordance with animal protocols approved by the Institutional Animal Use and Care Committee of WenZhou Medical College and performed according to the institutional ethical guidelines for animal experiment. After resuspension in PBS, MDA-MB-157 cells (5×10⁶ /mouse) were injected subcutaneously into the dorsal flanks of the nude mice. Ten days after implantation when the tumor became palpable at the size of ~5mm in diameter, intratumor injection with 50 µg of miR-155 ASO dissolved in 100 µL of DMEM mixed with 3µL of LipofectamineTM2000 was done twice a week (Li et al., 2009). The size of the tumor was measured every 5 days by a Vernier calliper along two perpendicular axes for a month. The volume of the tumor was calculated with the formula: volume (mm³) =0.5×width²×length. Tumor xenografts were harvested, weighted and used for immunohistochemistry for caspase-3.

Immunohistochemistry

For immunohistochemistry, rabbit polyclonal antibody to caspase-3 (Blue Gene, Shanghai) was used as primary anti-body for overnight incubation at 4°C. The sections were subsequently treated with biotinylated secondary antibody, followed by further incubation with streptavidin-

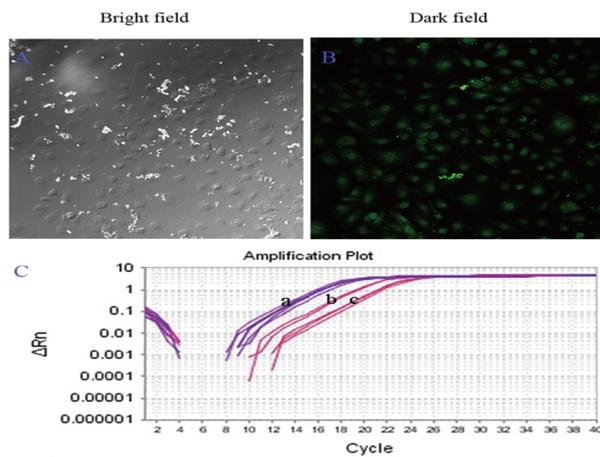


Figure 1. Transfection Efficiency Detected by Laser Confocal Microscope and miR-155 Expression in the Treated MDA-MB-157 Cells by Real-time PCR. (A) The bright field image (original magnification $\times 200$). (B) The dark field image showed most of the cells (more than 80%) were stained green fluorescent, indicating that transfection efficiency was higher than 80% (original magnification $\times 200$). (C) Representative amplification curves showed the CT value of miR-155 ASO group was higher than that of SCR group, indicating that miR-155 ASO down-regulated miR-155 levels in MDA-MB-157 cells. Reactions were done in triplicate. (a) 5s rRNA expression in MDA-MB-157 cells; (b) miR-155 of SCR group; (c) miR-155 of ASO group

horseradish peroxidase complex. Diaminobenzidine (Dako) was used as a chromogen and sections were lightly counterstained with hematoxylin. The proportion of caspase-3 immunostaining tumor cells varied from 0% to 100%, and a five-grade scoring system was used to evaluate the degree of immunostaining: score 0, 0 to 1%; score 1, 2% to 10%; score 2, 11% to 50%; score 3, 51% to 80%; score 4, 81% to 100% of tumor cells with positive immunostaining. And a four-grade scoring system was used to assess the dying strength: score 0, cytoplasmic without dying; score 1, faint yellow; score 2, claybank; score 3, sepia. The total score is the product of the degree of immunostaining and the dying strength: score 0 for negative (-); score 1~4 for weak positive (+); score 5~8 for positive (++); score 9~12 for strongly positive.

Statistical analysis

For all data, statistical analysis was performed in SPSS 17.0 for Windows (SPSS Inc.). The Independent Samples T-Test was applied to analyze the miR-155 ASO down-regulation of miRNA expression. The one-way ANOVA test was performed to investigate the differences in the obtained results of WST-8 array and apoptosis analysis. The difference in caspase-3 expression in tumor xenografts was examined by Kruskal-Wallis H test and the subsequent Nemenyi test for pairwise samples. All tests were two-tailed, and the significance level was set at $P < 0.05$.

Results

Transfection efficiency detection

Eight hours after transfection with 75nM 5'FAM SCR in MDA-MB-157 cells, the transfection efficiency was detected by laser confocal microscope. As shown

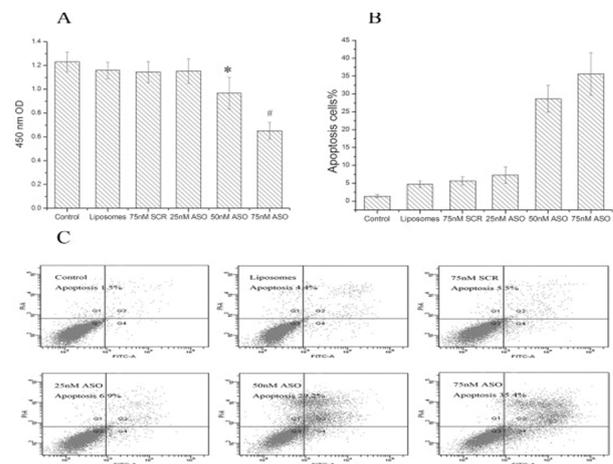


Figure 2. miR-155 ASO Show Growth Inhibition and Apoptosis Increase *in vitro*. (A) MDA-MB-157 cells were transfected with miR-155 ASO or miR-155 SCR. WST-8 assays were performed at the indicated time points after transfection. * $P=0.005$ compared to control group; # $P=0.000$ compared to control group. (B) MDA-MB-157 cells were transfected with miR-155 ASO or SCR for 6 hours, and incubated in medium containing 10% FCS for another 24 hours. Apoptosis of MDA-MB-157 cells were double stained with annexin V and PI, and detected by flow cytometry. 50nM and 75nM miR-155 ASO could efficiently induced apoptosis ($P < 0.01$ as compared with SCR). (C) The images of apoptosis were analysed by double staining with annexin V and PI

in Figure 1B, the vast majority of cells (more than 80%) contain fluorescent in the cytoplasm, which indicated the transfection efficiency was higher than 80%.

miR-155 ASO down-regulation of miRNA expression

To validate whether miR-155 ASO decreased miR-155 levels in treated MDA-MB-157 cells, miR-155 and 5s rRNA expression was determined by real-time RT-PCR as anteriorly described. As shown in the amplification curves (Figure 1C), the CT value of miR-155 ASO group was higher than that of SCR group, and the Δ CT value was 4.81 ± 0.20 and 3.04 ± 0.09 , separately. There was a statistical significance between the two groups ($t=11.1$, $P=0.00$), which showed that the level of miR-155 in MDA-MB-157 cells was down-regulated by miR-155 ASO.

miR-155 ASO inhibition of MDA-MB-157 cells Viability

In this study, we determined the influence of miR-155 ASO on cell viability by WST-8 array. Optical densities at 450 nm were obtained for 4 groups: 1.23 ± 0.08 for control group, 1.16 ± 0.07 for liposomes group, 1.15 ± 0.09 for SCR group and 1.15 ± 0.10 , 0.97 ± 0.14 and 0.65 ± 0.07 , respectively for 25 nM, 50 nM and 75 nM concentrations of miR-155 ASO (Figure 2A). These results demonstrated that optical density and therefore cell viability was similar in control, SCR, 25 nM miR-155 ASO and liposome groups ($P=0.31$, one-way ANOVA test). However, there were significant differences of optical density in 50nM miR-155 ASO and 75 nM miR-155 ASO group compared with control group ($P=0.00$, $P=0.00$, one-way ANOVA test, respectively). Optical density and cell viability gradually decrease with the increase of miR-155 ASO concentration (1.15 ± 0.10 , 0.97 ± 0.14 , 0.65 ± 0.07 , respectively). At 75nM concentration of miR-155 ASO,

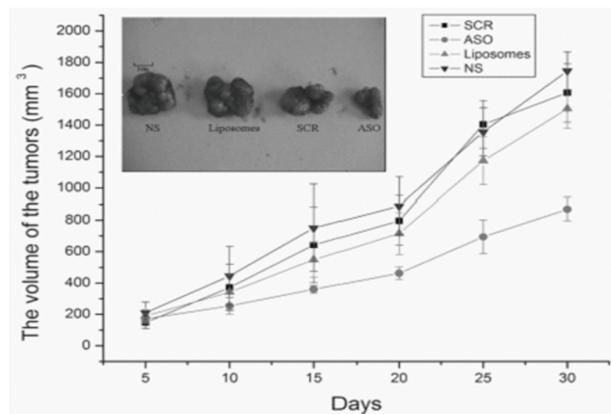


Figure 3. miR-155 ASO Restrains Tumor Growth of Breast Cancer Cells in Nude Mice. MDA-MB-157 cells were injected subcutaneously into the dorsal flanks of the nude mice and the tumors were treated with miR-155 ASO, SCR, Liposomes or normal saline biweekly. The curves show the time course of tumor growth, and the inset photographs are representative xenografted tumors 30 days after inoculation

optical density and cell viability were nearly half of these parameters in control group. These data indicated that a higher concentration of miR-155 ASO had a higher toxicity effect on MDA-MB-157 cells and could decrease the cell viability and proliferation.

miR-155 ASO promotion of MDA-MB-157 cells apoptosis

To explore the effects of miR-155 ASO on cells apoptosis, miR-155 ASO treatment was investigated in MDA-MB-157 cells. Apoptotic MDA-MB-157 cells were detected by double staining with annexin V and PI. The results demonstrated that miR-155 ASO could induce cell apoptosis. Along with the increase of concentration of miR-155 ASO, the apoptosis rate of MDA-MB-157 cells gradually increased (Figure 2B). The double stained images are shown in Figure 2C.

miR-155 ASO suppresses tumor growth in the nude mice

To further investigate the role of miR-155 in tumor growth, we assessed the effects of inhibition of miR-155 on the growth of xenograft tumors in vivo. Excepted one nude mouse in saline group, all the other nude mice were successfully transplanted and had tumor growth. In nude mice, tumor treated with miR-155 ASO (biweekly intratumor injection), but not with miR-155 SCR, LipofectamineTM2000 or normal saline, significantly reduced tumor size (Figure 3). When dissected at the end of the study (day 30), the average tumor weight of the miR-155 group and saline group were 0.79 ± 0.09 and 1.68 ± 0.12 , respectively, which indicated that the tumor suppressor rate was 52.9%.

miR-155 ASO up-regulation of caspase-3 in xenograft tumors

To further validate whether miR-155 ASO inhibits tumorigenesis of breast cancer xenografts by up-regulating a pivotal apoptosis regulatory factor, caspase-3, we performed immunohistochemistry for it. As expected, there were hardly any positive cells in xenograft tumors of saline group, while some positive cells were observed in

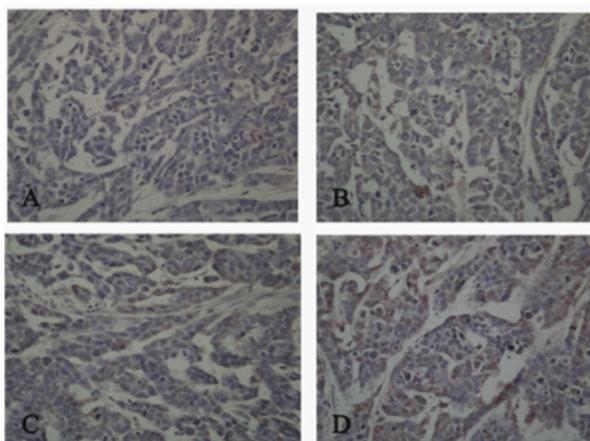


Figure 4. Representative Results of Immunohistochemical Staining of Caspase-3 in Xenograft Tumors of the Four Groups (original magnification $\times 400$). (A) Caspase-3 expression in cells of xenograft tumors of saline group is almost absolutely lost. (B) A few caspase-3 expression in liposomes group. (C) some positive cells were observed in 75 nM SCR group. (D) Caspase-3 was high expressed in 75 nM ASO group

liposomes group and 75 nM SCR group. However, in 75 nM ASO group, there were plenty of cells stained sepia, which indicated that most of the tumor cells were caspase-3-positive cells (Figure 4). The differences of expression of caspase-3 among the four groups were statistically significant ($\chi^2=15.2$, $P=0.00$), the subsequent Nemenyi test indicated that the score of 75 nM ASO group was markedly higher than the other three groups (saline group: $\chi^2=34.5$, $P<0.05$; liposomes group: $\chi^2=9.55$, $P<0.05$; 75 nM SCR group: $\chi^2=8.17$, $P<0.05$).

Discussion

As previous research indicated, miR-155 acts as one of multifunctional miRNAs in many pathophysiological process, such as immunology (Louafi et al., 2010; Oertli et al., 2011; Ghorpade et al., 2012), inflammation (O'Connell et al., 2010; Bhattacharyya et al., 2011; Busch and Zerneck, 2012), hematopoiesis (Vasilatou et al., 2010), angiocardopathy (Urbich et al., 2008; Yao et al., 2011) and carcinogenesis. It has been implicated in the promotion of tumor growth, proliferation, antiapoptosis, and response to chemotherapy (Kong et al., 2010). Diverse studies have shown that miR-155 is overexpressed in different tumor types. Taken together with the results presented here, a number of studies support the hypothesis that miR-155 might be one of the most relevant oncogene-like factors among the class of miRNAs.

In the present study, we utilized antisense-based technology, one of the most efficient tools with characteristics of high degree of specificity, high efficiency and low toxic side effects, for down-regulating the expression of the miRNA. Antisense oligonucleotides of DNA and RNA both inhibit miRNA function by pairing miRNA. However, because of DNA oligonucleotide degradation of pre-miRNAs, pre-miRNAs, and mature miRNAs through an RNase H cleavage, it leads to the alteration of miRNA levels in cells (Li et al., 2010). This change is easily and effectively validated by real-

time PCR or Northern blot. Therefore, we consider that antisense oligodeoxynucleotides as miRNA inhibitors have advantages compared with RNA oligonucleotides. In our research, miR-155 down-regulation by its antisense oligodeoxynucleotides has been confirmed by quantitative real-time PCR.

We chose MDA-MB-157 cells, which had been confirmed highly express miR-155, as our primary experimental material. And then we performed experiments to observe the change of biological behavior of them by using antisense technology. Transfection of MDA-MB-157 cells with either miR-155 ASO or SCR were done successfully by at least 80% efficiency. WST-8 test was performed and the results indicated that although 25nM and 50nM of miR-155 ASO have toxic effect on MDA-MB-157 cells, 75nM of miR-155 ASO could repress tumor cell proliferation strongly. Moreover, apoptosis analysis demonstrated that the apoptosis rates of the group of 50nM and 75nM of miR-155 ASO were significantly higher than the other three groups. This result is consistent with the findings of our previous research which was experimented with HS578T cells, another cell line with miR-155 highly expressed (Kong et al., 2010). One possibility could be the similar potency of miR-155 ASO that is specific to miR-155-expressing cancer cells.

To further determine whether miR-155 regulates tumor growth in vivo, we used tumor xenografts by inoculating MDA-MB-157 cells with modulated miR-155 expression in nude mice. Although the tissue structure and cell morphology of MDA-MB-157 xenografts treated with miR-155 ASO were not different from those treated with miR-155 SCR, LipofectamineTM2000 or saline alone, injection with miR-155 ASO tremendously reduced the tumor size and tumor weight (Figure 3). This is in part consistent with the findings of Jiang S et al (Jiang et al., 2010). These findings in breast cancer xenografts suggest that antagonizing miR-155 may hold great promise for designing novel therapeutic strategies against breast cancers. Additionally, we found that injection of miR-155 ASO could enhance the expression of caspase-3 determined by immunohistochemical staining (Figure 4). Thus, our findings suggested that blockade of miR-155 retards xenografts development in vivo, probably by inducing apoptosis of cancer cells via caspase-3 up-regulation. It should be noted that caspase-3 is required for some typical hallmarks of apoptosis and is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types examined (Porter and Jänicke., 1999). In a study of intervertebral disc degeneration, researchers found that up-regulation of miR-155 resulted in repression of caspase-3, whereas knockdown of miR-155 led to over-expression of caspase-3. Further studies conclusively showed that caspase-3 was identified as a novel target of miR-155 (Wang et al., 2011). Although Ovcharenko D et al (Ovcharenko et al., 2007) reported that up-regulation of miR-155 decreases TNF-related apoptosis-inducing ligand (TRAIL)-induced caspase-3 activity in MDA-MB-453 cells via a yet-undefined mechanism, our in vivo modulation of miR-155 in breast cancer xenografts provides beneficial points of clinical relevance.

In conclusion, our study indicates that MDA-MB-157

cells transfected with miR-155 ASO show growth inhibition and apoptosis increase in vitro. Moreover, miR-155 ASO can significantly repress tumor growth in vivo, presumably by inducing apoptosis via caspase-3 up-regulation. Therefore, our data suggest that miR-155 may serve as an potential and effective target for the development of novel anticancer therapies.

Acknowledgements

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