HIF-1α decoy oligodeoxynucleotides inhibit HIF-1α signaling and breast cancer proliferation

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Abstract. Although HIF-1α is considered an attractive target for the development of cancer therapies, like other transcriptional factors, it has been regarded as 'undruggable'. The decoy approach is a new class of antigene strategy that can be used to modulate the function of endogenous transcriptional factors. Here, we designed a decoy oligodeoxynucleotide (ODN) and tested its effect on the function of HIF-1α. We found the HIF-1α decoy ODN could efficiently enter into cells. Furthermore, these decoy ODNs can significantly block the expression of VEGFA, a known targeted gene of HIF-1α suggesting that the HIF-1α decoy ODNs can inhibit the function of HIF-1α. More importantly, the HIF-1α decoy ODN induced apoptosis and cell cycle arrest in MDA-MB-231 breast cancer cells. In summary, HIF-1α decoy ODNs can inhibit the function of HIF-1α and induce cancer cell apoptosis. Therefore, HIF-1α decoy ODNs should be further modified to improve their biological activity *in vivo*.

Introduction

Breast cancer originates from breast tissue and can be classified into ductal and lobular carcinomas depending on its origin (1,2). Breast cancer is the most common invasive cancer in women and is the cause of 13.7% of cancer-related deaths in women (3,4). Although great advances in treatment and detection have been made, the overall survival rate for breast cancer is still low (4,5). In particular, patients with triple-negative breast cancers have the most adverse prognosis with a 5-year survival rate <30% (6,7). This statistic highlights the urgent need to develop novel therapeutic targets and strategies for the eradication of breast cancer.

The tumor microenvironment is the cellular environment in which the tumor cells exist. It consists of tumor cells, surrounding blood vessels, immune cells, fibroblasts, signaling molecules and the extracellular matrix (8,9). The tumor microenvironment is characteristically hypoxic (10). As the tumor mass increases, the interior of the tumor moves farther away from existing blood supply and hence becomes hypoxic. Genetic instability is a major consequence of the hypoxic environment and contributes largely to the tumor heterogeneity. Hypoxia also causes the upregulation of hypoxia-inducible factor 1 α (HIF-1α), which induces angiogenesis and is associated with poor prognosis and the activation of genes associated with metastasis, which is the major cause of cancer-related deaths (11,12). Due to the critical role of HIF-1α in the tumor development, it has been considered an attractive target for the development of cancer therapies (13,14). To date, however, strategies that can be used to inhibit the function of HIF-1α remain elusive.

Despite mounting evidence implicating transcription factors such as HIF-1α in carcinogenesis, these proteins have been regarded as undruggable. The decoy approach is a new class of antigene strategy that can be used to modulate the function of cellular transcription factors (15). It is essentially a synthetic double-stranded cis-element oligodeoxynucleotide (ODN), which can be recognized by transcriptional factors. These oligodeoxynucleotides also contain chemical modifications such as phosphorothioation to increase their stability (16,17). Theoretically, this approach gives a new way to modulate the activity of all transcription factors. However, this strategy has only been tested on a handful of transcription factors such as NF-κB and STAT3 (18,19). Recently, STAT3 decoys were used in a phase 0 clinical trial, which highlights the potential of this therapeutic approach in the clinic (20). Given the important role of HIF-1α in cancer development, the use of the decoy approach to inhibit its function might prove to be a useful therapeutic tool. However, to our knowledge, there is only one report to date showing that the function of HIF-1α can be inhibited by decoy ODNs in oral squamous cell carcinoma cells (21). Herein, we provide *in vitro* evidence and demonstrate that the function of HIF-1α can be inhibited by decoy ODNs in breast cancer, a common cancer in women.

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Abbreviations: ODN, oligodeoxynucleotide; TFs, transcription factors; HIF-1α, hypoxia inducible factor-1α; VEGFA, vascular endothelial growth factor A; BCSCs, breast cancer stem cells

Key words: decoy strategy, HIF-1α signaling, BCSC, apoptosis, cell cycle, triple-negative breast cancer, hypoxia

Materials and methods

Cell culture and flow cytometry analysis. The human MDA-MB-231 breast cancer cell line was obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) at 37˚C and 5% CO_2. For suspension cultures, cells were cultured in serum-free DMEM/F12 (Gibco) containing 20 ng/ml epidermal growth factor (EGF; PeproTech Asia, Rehovot, Israel), 20 ng/ml basic fibroblast growth factor (bFGF; PeproTech Asia), 2% B27 (Invitrogen, Carlsbad, CA, USA) and 0.4% bovine serum albumin (Beyotime, Wuhan, China) at a density of 1,000 cells/ml. Suspension cells were grown in T-75 culture flasks or a 6-well plate with ultra low attachment surface at 37˚C and 5% CO_2. Tumor spheres were passaged every 3 days at 1,000 cells/ml following mechanical dissociation with a 40-μm cell strainer.

To identify $CD44^+CD24^{-/low}$ cells in the MDA-MB-231 cell line, we measured expression of $CD44^+CD24^{-/low}$ using a fluorescence-activated cell sorting (FACS)-Vantage SE instrument (Becton-Dickinson, Franklin Lakes, NJ, USA). The antibodies used were phycoerythrin (PE) labeled anti-CD24, fluorescein isothiocyanate (FITC) labeled anti-CD44 and their corresponding isotype controls (BioLegend, San Diego, CA, USA). Cells were harvested and gently disassociated to form a single cell suspension. Staining was made according to the manufacturer's protocol.

HIF-1α decoy oligonucleotide synthesis and preparation. HIF-1α specific decoy oligonucleotides (ODNs) containing either the wild-type HIF-1 transcription factor binding sequence or a mutant sequence as control were synthesized. No sequence homology was found between the synthesized nucleotide sequence and the known transcription factors. The sequences of the HIF-1α decoy ODNs were as follows: 5'-GCCCTACGTG CTGTCTCA-3' (sense); 5'-TGAGACAGCACGTAGGGC-3' (antisense). The sequences of the mt-HIF-1α decoy ODNs sequence were as follows: 5'-GCCCTTACAACTGTCTCA-3' (sense); 5'-GAGACAGTTGTAAGGGC-3' (antisense). ODNs were labeled by FAM for the direct monitor after transfection. All oligonucleotides were synthesized by Life Technology (Guangzhou, China).

Hypoxia induction. The cobalt dichloride chemical method was used to simulate a hypoxic environment. Cells were pretreated with cobalt dichloride (Sigma-Aldrich, St. Louis, MO, USA; dissolved in ultrapure water at 100 mmol/l and stored at -20˚C) at a concentration of 100 μmol/l for 12 h before gene transfection.

Gene transfection. MDA-MB-231 cells were seeded at a density of 10^5 cells/ml per well in 6-well plate. Transfections were performed when cells reached 40-50% confluence using Lipofectamine® LTX & PLUS™ reagent (Invitrogen) according to the manufacturer's instructions. Briefly, the culture media was replaced with Opti-MEM® reduced serum medium (Invitrogen). Liposomes (5 μl) were diluted into 250 μl Opti-MEM® reduced serum medium and dsDNA (100 pmol) with 1 μl PLUS™ reagent was added into the mix. Following a 20-min incubation, the liposome complexes were added to the cells. After incubation with the cells for 4 h, the liposome complexes were removed and the complete medium was added to the cells.

Table I. Primer sequences for human target genes.

Target genes	Primer sequences
HIF-1α	F: 5'-GGCGCGAACGACAAGAAAAAG-3'
	R: 5'-CCTTATCAAGATGCGAACTCACA-3'
VEGFA	F: 5'-CAGCGCAGCTACTGCCATCCAATCGAGA-3'
	R: 5'-GCTTGTCACATCTGCAAGTACGTTCGTTTA-3'
GADPH	F: 5'-CAGCCTCAAGATCATCAGCA-3'
	R: 5'-TGTGGTCATGAGTCCTTCCA-3'

Microscopy studies. An inverted phase contrast fluorescence microscope (Olympus, Tokyo, Japan) was used to observe the transfection efficiency after a 6-, 12-, 24-, 36- and 48-h transfection. MDA-MB-231 cells growing on slides were stained with DAPI 24 h after transfection and observed using confocal laser scanning microscope (Zeiss LSM 510, Jena, Germany).

Quantitative PCR. MDA-MB-231 cells were collected at 6, 12, 24, 36 and 48 h post-transfection. Total RNA was extracted using TriPure (Roche, Basel, Switzerland). The SuperScript™ First-Strand Synthesis system (Invitrogen) was used to synthesize first-strand cDNA from total RNA. Real-time PCR was carried out using the SYBR Green I reagent (Invitrogen) on Mx3000P (Agilent Technologies, Palo Alto, CA, USA). The mRNA level was analyzed by $2^{-\Delta\Delta Ct}$ values. The primer sequences used are shown in Table I.

Western blot analysis. MDA-MB-231 cells were collected at 6, 12, 24, 36 and 48 h after transfection and lysed in RIPA lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 100 mg/ml PMSF (Beyotime). Protein concentration was measured by the BCA method (Pierce, Rockford, IL, USA). Total proteins were separated by SDS-PAGE (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes (Millipore, Boston, MA, USA). Following blocking with 5% non-fat milk, membranes were probed with HIF-1α, VEGFA antibodies (CST, Boston, MA, USA) at 4˚C for 12 h and incubated with horseradish peroxidase-conjugated secondary antibody (CST). The signals were visualized using an electrochemiluminescence kit (Millipore) and then exposed to X-ray film (Kodak, Rochester, NY, USA). The bands were scanned using Bio-5000Plus (Microtek, Xinzhu, Taiwan) and quantitation was determined as the optical densities of the bands using IPP Image software 6.0 (Media Cybernetics, Bethesda, MD, USA).

Apoptosis analysis. Firstly, the morphology of the apoptotic cell was observed under a microscope. In brief, MDA-MB-231 cells were fixed by methanol at 24 h post-transfection, washed

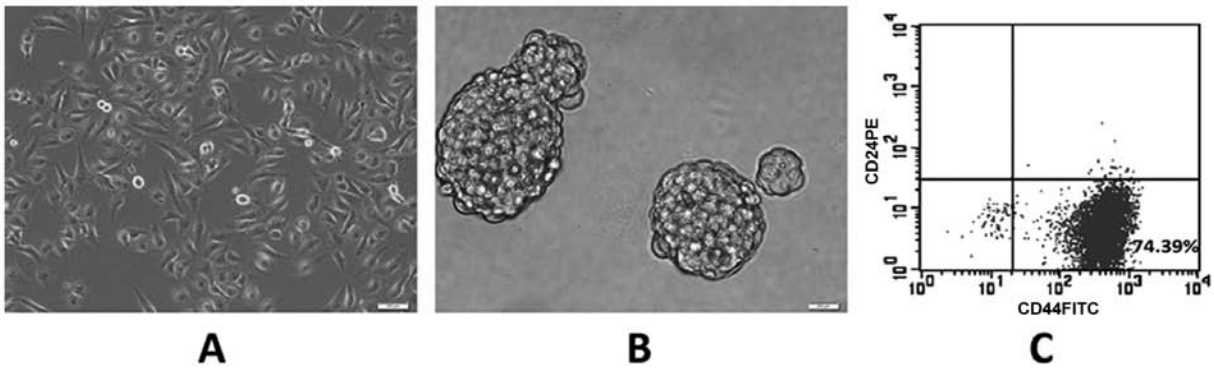

Figure 1. MDA-MB-231 cells form microspheres in suspension cultures. (A) Representative image of MDA-MB-231 cells cultured in adherent conditions. (B) Representative image of floating microspheres after 6 days in a suspension culture; scale bar, 200 μm. (C) $CD44^{+}CD24^{-/low}$ population in MDA-MB-231 cell line.

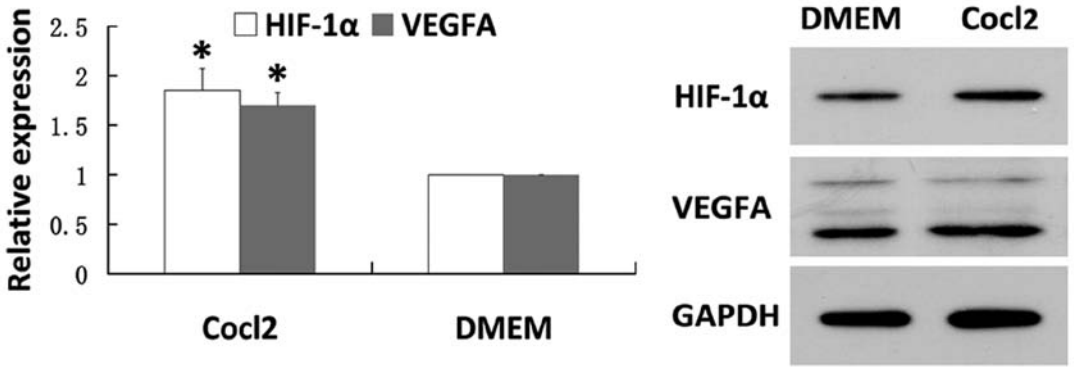

Figure 2. Expression of HIF-1α and VEGFA in MDA-MB-231 cells. Cells were treated with cobalt dichloride (100 μmol) for 24 h. Total RNA and protein were extracted and gene expression was analyzed. QPCR analysis (left) was performed to measure transcript levels of HIF-1α and VEGFA. Western blot analysis (right) was performed to determine the protein expression of HIF-1α and VEGFA. Untreated cells were used as the control ($^{*}p<0.05$).

in PBS, and stained with Hoechst 33258 (Sigma-Aldrich). After a 20-min incubation at 37˚C, apoptotic nuclei were observed under fluorescence microscopy (Olympus, Tokyo, Japan).

Secondly, apoptosis assay was performed using Guava Nexin reagent (Millipore) according to the manufacturer's instructions. In brief, cells were collected at 24 h post-transfection and adjusted at a density of $2x10^{5}$-$1x10^{6}$ cells/ml. Cells (100 μl) were incubated in the dark with 100 μl Guava Nexin Reagent for 20 min at room temperature. The cells were then analyzed using flow cytometry (Guava System, Millipore).

Thirdly, the cell cycle was investigated using Guava cell cycle reagent (Millipore) according to the manufacturer's instructions. In brief, transfected cells were collected, washed with PBS, centrifuged at 450 g for 5 min, and fixed in 500 μl PBS containing pre-cold 70% ethanol at 4˚C for 72 h. Cells were then washed in PBS and incubated with Guava cell cycle reagent for 30 min. At this time, cells were passed through a 40-μm cell strainer and analyzed by flow cytometry (Guava System, Millipore).

Statistical analysis. Statistical analysis was performed using SPSS software (version 13.0, SPSS Inc., Chicago, IL, USA). The results are presented as mean ± standard deviation (mean ± SD). Differences in means between groups were analyzed for significance using Student's t-test or ANOVA as appropriate. A p-value <0.05 was considered statistically significant.

Results

Enrichment of a subpopulation of putative breast cancer stem cells (BCSCs) in MDA-MB-231 cells and microspheres generated under suspension conditions. MDA-MB-231 cells grow as adherent cultures in culture medium with serum (Fig. 1A). Given that $CD44^{+}CD24^{-/low}$ cells are considered to be BCSCs, we measured the cell surface expression of these markers by FACS analysis to determine whether there is a subpopulation of BCSCs in MDA-MB-231 cultures. Interestingly, we found that 71.52-74.39% of the cell population was $CD44^{+}CD24^{-/low}$ (Fig. 1C). Accordingly, when MDA-MB-231 cells were adapted to serum-free medium culture conditions, we found that large spherical aggregates 60-200 μm in diameter formed within the first 6 days in culture (Fig. 1B). Together, these results suggest that MDA-MB-231 cells contain a subpopulation of cells with stem-like properties.

HIF-1α and VEGFA expression is induced under hypoxic conditions. Under normal culture conditions, MDA-MB-231 cells express low levels of both HIF-1α and VEGFA as shown

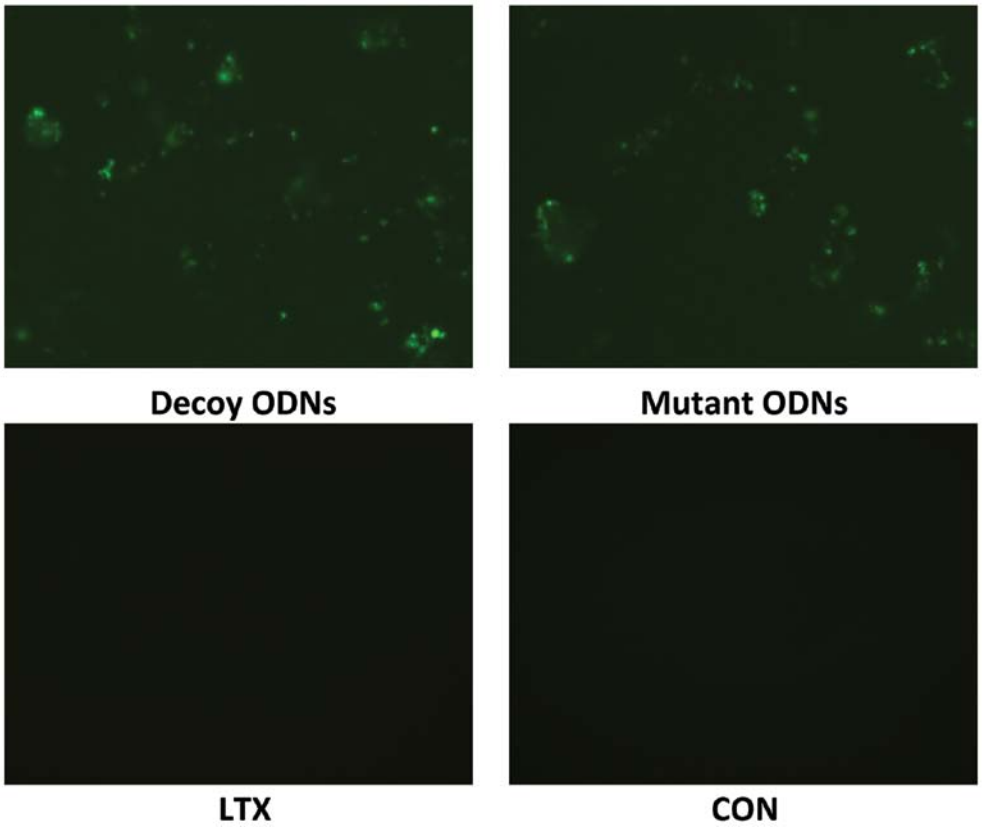

Figure 3. Expression of ODNs in MDA-MB-231. Transfected cells were observed at 12 h after transfection under an inverted phase contrast fluorescence microscope. Intracellular fluorescence could be detected as early as 6 h after transfection and last for >48 h after transfection. The signal was relative weaker compared to GFP (x200).

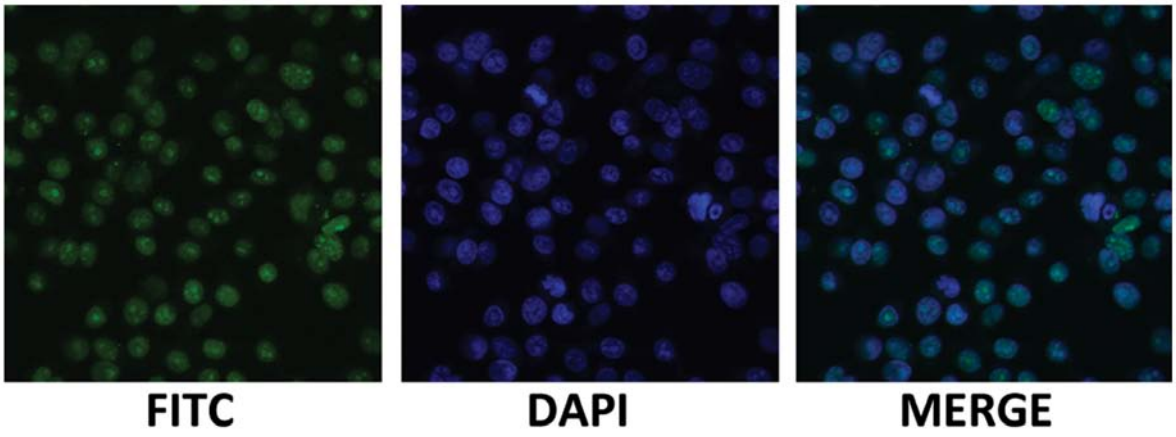

Figure 4. ODNs localize to the nucleus in MDA-MB-231 cells. Transfected cells stained with DAPI (blue) and examined by confocal microscopy (x400).

in Fig. 2. When MDA-MB-231 cells were treated with $CoCl_2$, a hypoxia inducer, we saw a corresponding increase in the expression levels of HIF-1α and VEGFA at both the mRNA and protein levels as shown in Fig. 2. These results indicate that MDA-MB-231 cells are a suitable model system to study the function of HIF-1α and VEGFA in normal and hypoxic culture conditions.

HIF-1α decoy ODNs can translocate into the cells and localize to the nucleus. Although HIF-1α is an ideal target for the development of anticancer therapies, to date there are no strategies in place to inhibit its activity. To test whether decoy ODNs are an effective approach to inhibit HIF-1α activity, we first determined the intracellular distribution of HIF-1α decoy ODNs labeled using fluorescein amidite (FAM). We found that both the decoy ODNs and the mutant ODNs were taken up by the cells as early as 6 h post-transfection (Fig. 3). Decoy ODNs persisted inside the cells for several hours but their fluorescence signal decreased with time as expected (Fig. 3). Confocal imaging analysis confirmed that the majority of the decoy ODNs were distributed to the nucleus (Fig. 4).

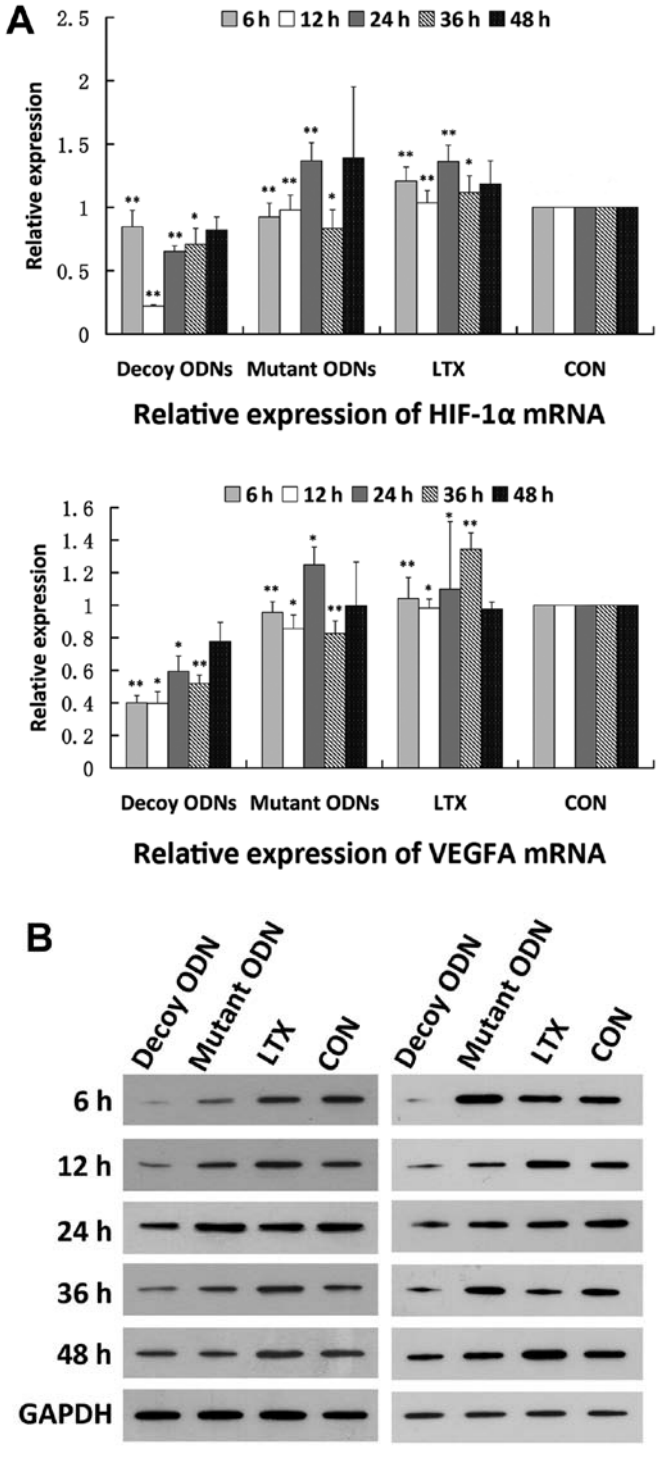

Figure 5. Downregulation of HIF-1α and VEGFA expression by decoy ODNs. MDA-MB-231 cells were transfected with decoy or mutants ODNs and collected at the indicated time-points. Total RNA and protein were extracted. QPCR were used to detect HIF-1α and VEGFA mRNA levels ($^{*}p<0.05$, $^{**}p<0.01$). Western blot analysis was performed to detect HIF-1α and VEGFA protein expression levels (LTX, cells treated with cationic liposomes LTX only; control, cells treated with PBS only and ODNs).

HIF-1α decoy ODNs inhibit the expression of VEGFA. Next, we determined whether HIF-1α decoy ODNs could inhibit the activity of HIF-1α by competing for its binding sites. We found that HIF-1α decoy ODNs had a minimal effect on the expression levels of HIF-1α itself as shown in Fig. 5. In contrast, the HIF-1α decoy ODNs significantly inhibited the expression of VEGFA both at the mRNA and the protein levels as shown in Fig. 5. Given that VEGFA is a direct target gene of HIF-1α (22), we reasoned that the HIF-1α decoy ODNs have the ability to inhibit the transcriptional activity of HIF-1α.

HIF-1α decoy ODNs promote apoptosis. Since HIF-1α is important for the survival of cancer cells and the decoy ODNs can inhibit HIF-1α-mediated transcriptional activities, we next asked whether treatment with the HIF-1α decoy ODNs tested could induce apoptosis. Nuclear staining of treated cells revealed that ODNs induced apoptosis within 24 h (Fig. 6) and the percentage of apoptotic cells was found to be significantly higher in the ODN group than in the control groups (Fig. 7).

HIF-1α decoy ODNs induce cycle arrest. To further study the biological effect of HIF-1α decoy ODNs, we analyzed the cell cycle distribution of cells after treatment with decoy ODNs. We found that HIF-1α decoy ODNs caused a cell cycle arrest at the G1 phase (Fig. 8). Taken together, our results suggest that HIF-1α decoy ODNs are translocated into the nucleus where they can inhibit HIF-1α-mediated transcriptional activities.

Discussion

Breast cancer is one of the most commonly diagnosed cancers and accounts for 22.9% of all cancer diagnoses. It has been reported that breast cancer was responsible for 458,503 deaths worldwide in 2008 alone (23). Traditionally, breast cancer subtypes are characterized by the expression of three cell surface receptors: estrogen receptor (ER), progesterone receptor (PR) and Her2/neu. Triple-negative breast cancer is a subtype of breast cancer that lacks the expression of all three receptors (7,24,25). In the clinic, the current therapeutic tools for breast cancer target the expression of these three receptors. Therefore, treatment of triple-negative breast cancers remains challenging.

MDA-MB-231 is a well-studied breast cancer cell line with highly aggressive characteristics that lacks the expression of the ER, PR and Her2/neu receptors. BCSCs are a subpopulation within breast cancer cells that are $CD44^{+}CD24^{-/low}$ (26). CSCs play a significant role in tumorigenesis, metastasis, and recurrence (27). Interestingly, we found that the majority of MDA-MB-231 cells were $CD44^{+}CD24^{-/low}$, which suggests that MDA-MB-231 is a suitable model to identify new therapeutic strategies for triple-negative breast cancers and to better understand the activity of BCSCs.

HIF-1α is the master transcription factor in hypoxia and plays a critical role in the development of cancer. Until now, there is no effective approach to inhibit its activity. The decoy approach is a novel technology which can be used to target the activity of transcription factors. Herein, we provide *in vitro* evidence to show that HIF-1α transcriptionally-mediated activities can be inhibited by decoy ODNs in culture.

Decoy ODNs constitute a new class of antigene strategy that utilizes modulation of endogenous transcriptional regulation. In essence, these synthetic double-stranded cis-element oligodeoxynucleotides compete with the endogenous

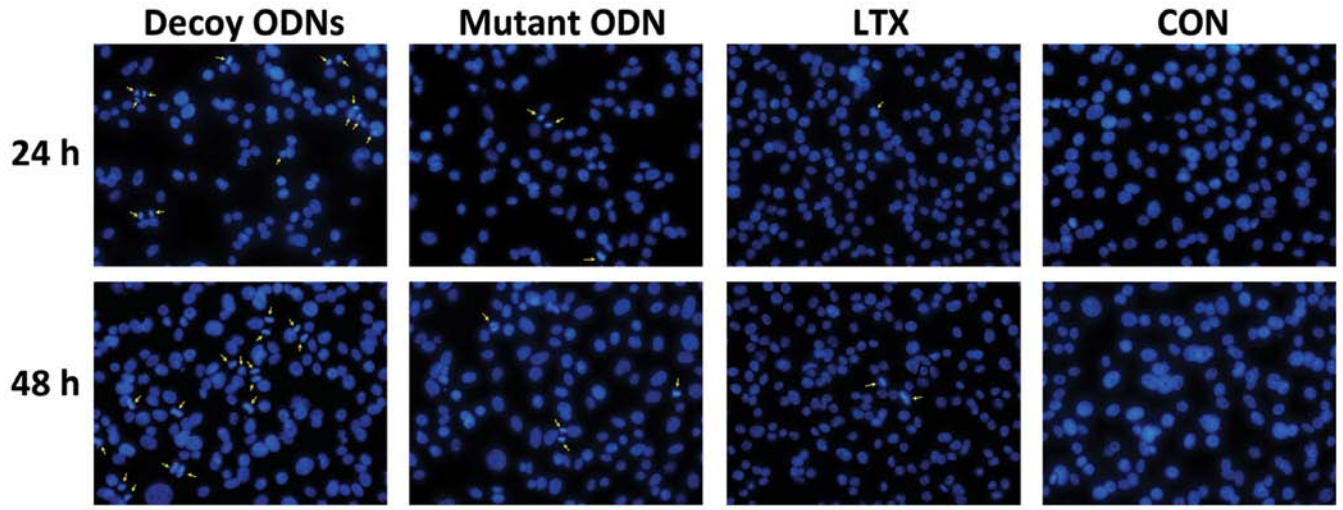

Figure 6. MDA-MB-231 cells exhibit apoptotic changes following transfection with ODNs. MDA-MB-231 cells were transfected with decoy ODNs, mutant ODNs and LTX and harvested at 24 and 48 h. Apoptotic morphology changes were observed by fluorescence microscopy (x400).

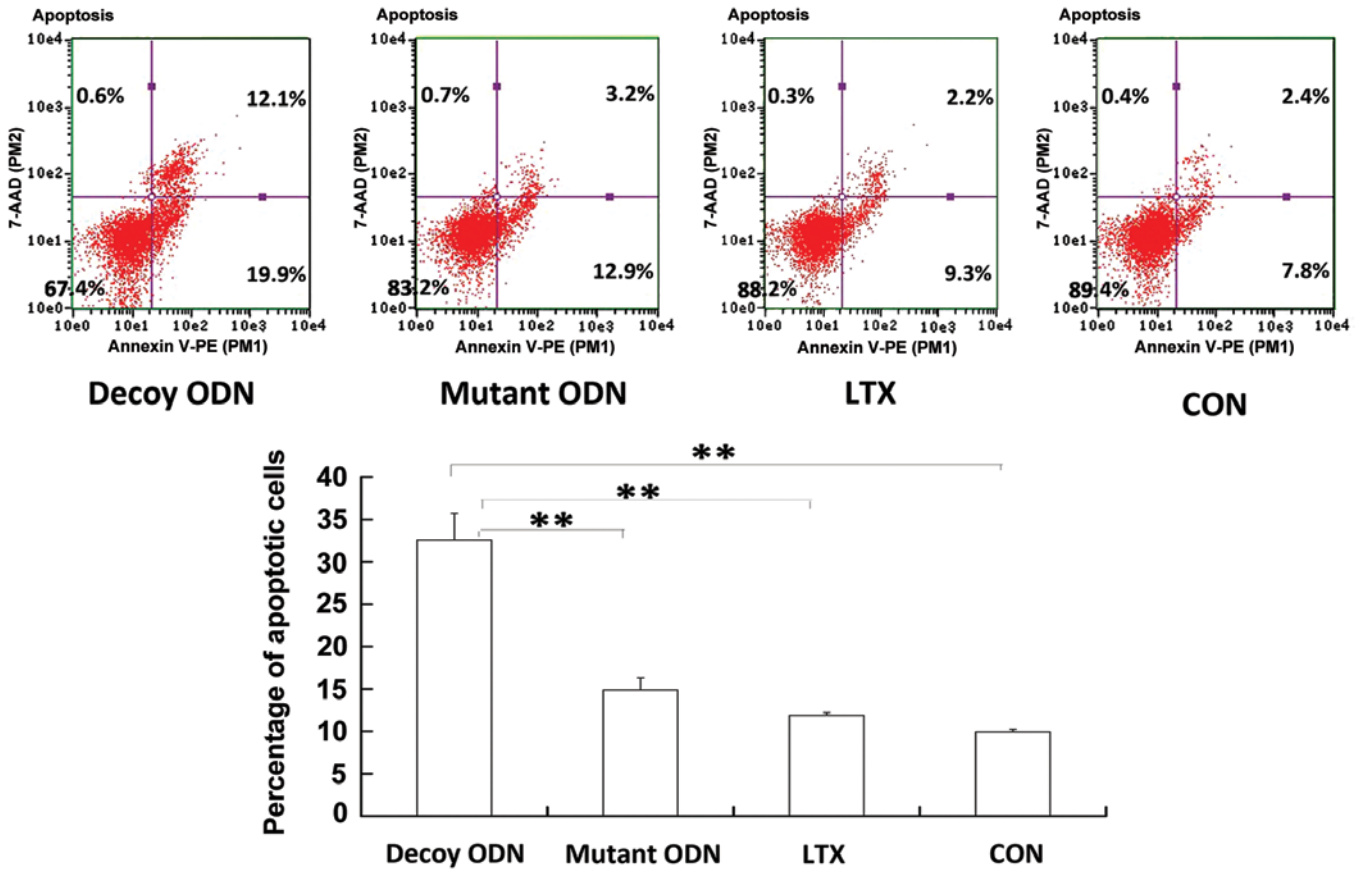

Figure 7. ODNs cause apoptosis in MDA-MB-231. Trasfected cells were harvested 24 h after transfection and examined by flow cytometry to determine the percentage of apoptotic cells in each group ($^{*}p<0.05$, $^{**}p<0.01$).

transcription factors for the binding to the DNA promoters (16,28,29). Therefore, if we know the DNA sequence of a certain transcription factor, we can design the decoy oligodeoxynucleotide to block the function of the transcription factor. To date, however, there are only a few transcription factors such as nuclear factor κB (NF-κB) (30), which have been tested by this technique. The information about HIF-1α is very limited. To our knowledge, however, there is only one report to date showing that decoy ODNs can be used to target HIF-1α in oral squamous cell carcinoma cells (21). Here, we provide convincing evidence that HIF-1α can be targeted by decoy ODNs also in breast cancer cells.

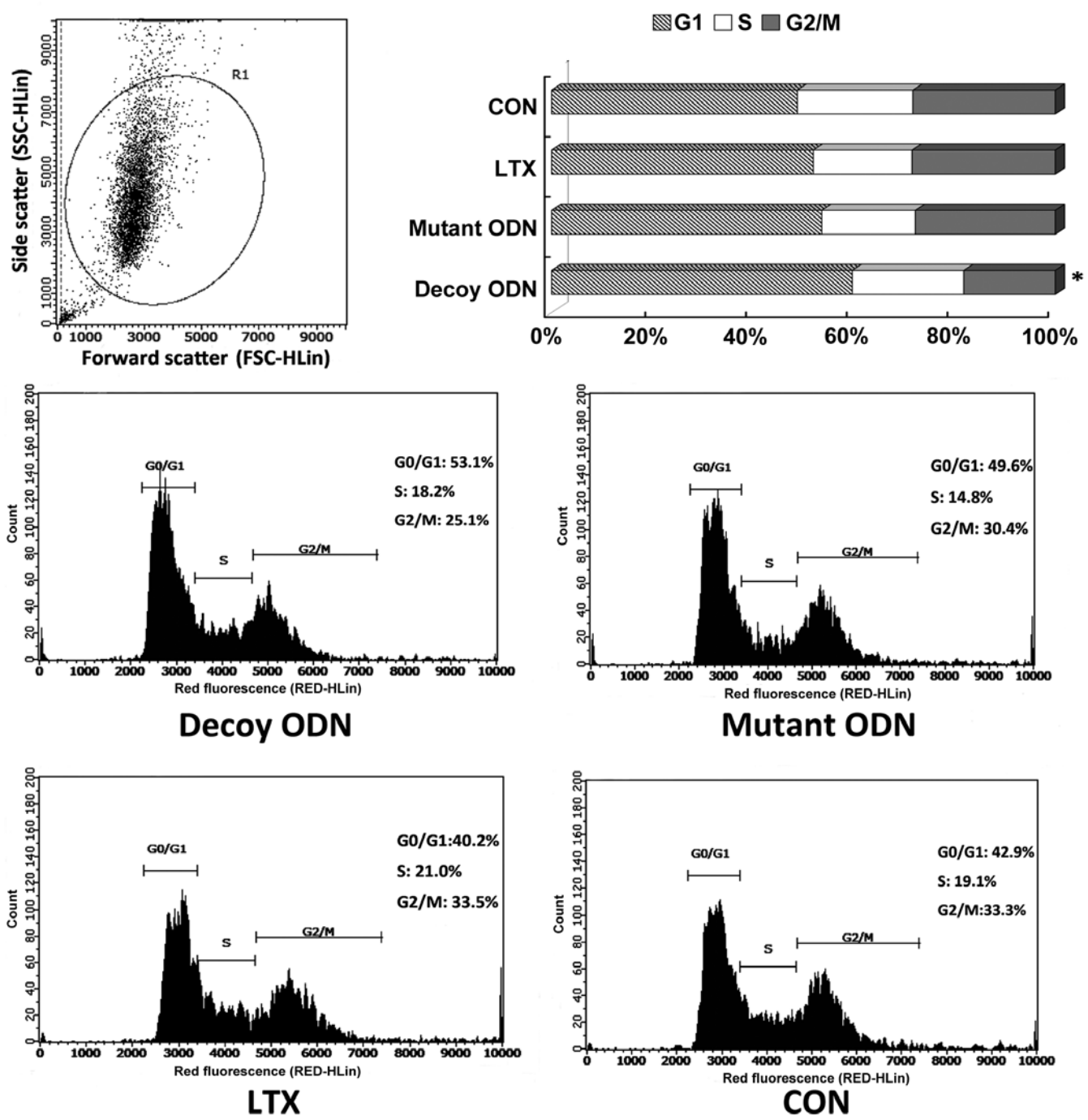

Figure 8. ODNs cause cells to arrest in the G1 phases of the cells cycle. MDA-MB-231 cells were trasfected with decoy ODNs, mutant ODNs and LTX and examined after 24 h using flow cytometry. Cells transfected with the decoy ODNs are arrested in the G1 phases of the cell cycle ($^{*}p<0.05$).

Usually, decoy ODNs are unstable *in vivo*. Chemical modifications such as phosphorothioation are therefore utilized to increase the stability for their use in cancer therapy. To date, one STAT3 decoy ODN is in phase 0 clinical trials (20). To improve the stability of the decoy ODNs *in vivo*, the decoy ODNs were modified by linking the oligonucleotide strands with hexaethylene glycol spacers. In future studies, this modification should be tested on the HIF-1α decoy ODNs to determine whether their biological effects are improved. In conclusion, here we report for the first time that HIF-1α activity can be effectively inhibited by decoy ODNs in breast cancer cells.

Acknowledgements

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