

Original Article

Aptamer-guided targeting of the intracellular long-noncoding RNA *HOTAIR*

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Abstract: Long non-coding RNAs (lncRNAs) are increasingly recognized as promising targets in cancer treatment. However, compared to targeting the ordinary protein-coding genes, suppressing non-coding RNAs expressed in cancer cells has been a more challenging task. The major hurdles lay on the requirement of a tumor-specific delivery system for the designated inhibitor to suppress the target transcripts within the cellular compartment. EGFR is a cancer driver gene which is frequently associated with the triple-negative phenotype of breast cancer. Prior studies have shown that expression of the tumor-promoting lncRNA *HOTAIR* (*HOX antisense intergenic RNA*) is positively regulated by the epithelial growth factor receptor (EGFR) in triple-negative breast cancer (TNBC), and consistently the expression of both genes is closely correlated in breast cancer. Here we show that a chimeric aptamer recognizing the epithelial growth factor receptor (EGFR) coupled with a siRNA against *HOTAIR* (EGFR aptamer-coupled *siHOTAIR*) preferentially and effectively down-regulated *HOTAIR* in EGFR-expressing cancer cells. Functionally, the EGFR aptamer-coupled *siHOTAIR* more potently inhibited the growth, migration, and invasion of EGFR-expressing TNBC cells as well as cells with reconstituted EGFR compared to cancer cells with low EGFR expression. Our results demonstrate a novel strategy of targeting cancer progression by aptamer-directed delivery of anti-lncRNA RNA interference that can be applicable to other cellular contexts and cancer types.

Keywords: EGFR, receptor tyrosine kinase, long non-coding RNA, *HOTAIR*, aptamer, triple-negative breast cancer, TNBC, siRNA

Introduction

Triple-negative breast cancer (TNBC), a major breast cancer subtype accounting for 10%-20% of breast cancer, is characterized by the lack of estrogen receptor, progesterone receptor, and the receptor tyrosine-protein kinase erbB-2 (ErbB2/HER2) [1, 2]. TNBC is characterized by promoted tumor growth and treatment resistance, and is often accompanied by enhanced aggressiveness. Due to the lack of conventional therapeutic targets, TNBC is the only subtype of breast cancer for which no FDA-approved targeted therapies are available. Currently chemotherapy is the main therapeutic option for TNBC, but resistance to the treatment fre-

quently occurs in relapsed advanced tumors. Blockade of immune checkpoint is emerging as a promising intervention for certain TNBC. However, the response of patients with metastatic TNBC to PD-1/PD-L1 blockade has been low, with response rates around 5% [3-5]. Thus, developing effective therapeutic approaches to TNBC remains an unmet medical need with significant clinical demands.

Expression of the receptor tyrosine kinase EGFR occurs in about 50% of TNBC tumors and therefore has been viewed as a promising target of TNBC [6]. Activation of EGFR has profound effect in cancer biology with multiple pathways such as MAPK, PI3K/AKT, and Wnt/ β -

catenin as the downstream effectors of activated EGFR for tumor promotion [6-8]. Mounting evidence derived from the clinic and *in vitro* studies supports that inhibition of EGFR promotes cell death, suppresses cancer cell growth, and enhances tumor progression, indicating a critical role in breast cancer biology [6, 9, 10]. However, the outcome of clinical trials for the therapeutic efficacy of targeting EGFR as single therapy in breast cancer has been disappointing due to intrinsic insensitivity or acquired resistance [11, 12], suggesting that targeting EGFR alone is unlikely to have major therapeutic impact in advanced breast cancer.

We previously showed that in TNBC cells EGFR activation induced nuclear translocation of the oncogenic transcription factor β -catenin which in turn induced gene expression of the lncRNA *HOTAIR* through two LIF1/TCF4-binding sites [13]. *HOTAIR* is a 2.3-kb transcript transcriptionally derived from the intergenic region of the *HOXC* homeotic gene cluster [14], and was the first lncRNA identified with tumor-promoting function whose expression is correlated with poor prognosis in breast cancer [15, 16]. *HOTAIR* forms a platform assembling epigenetic moderators to regulate gene expression. In addition, it has been reported that *HOTAIR* can regulate miRNA to promote cancer cell growth and migration [17, 18]. Expression of *HOTAIR* enhanced the growth and metastasis of xenograft tumors of mammary fat pad [15]. We have identified the chondroitin sulfate sulfotransferase CHST15 as one of the *HOTAIR*-regulated genes [19]. CHST15 modified the context of glycan modifications of cell surface proteins by increasing the contents of the chondroitin 4,6-disulfate which in turn promote cell migration, invasion, and tumor growth. Importantly, downregulation of *HOTAIR* resulted in loss of sensitivity of TNBC to EGFR inhibitors [13]. Thus, *HOTAIR* as well as the biological functions regulated by *HOTAIR* are critical tumor-promoting mechanism of the EGFR cascade in TNBC cells, suggesting that the *HOTAIR* lncRNA is a promising target in EGFR-expressing TNBC cells.

Aptamers are short (arbitrarily defined up to ~200 nucleotides) single-stranded nuclease-stabilized oligonucleotide. Through a series of selecting cycles (namely the selection processes of systematic evolution of ligands by expo-

nential enrichment; SELEX), the enriched aptamers specifically recognizing a molecular target can be isolated [20, 21]. Successful aptamers have a dynamic three dimensional conformation which specifically recognizes the designated target in a mode similar to antibodies, with the biological advantages of high tissue penetration, low immunogenicity, and long *in vivo* stability [22-24]. With this strategy, a series of EGFR-targeting aptamers have been developed [25-27]. The EGFR aptamer E07 (hereafter referred to as *aptEGFR*) binds to EGFR with high affinity ($K_d = 2.4$ nM), specifically targets EGFR-expressing breast cancer cells, blocks EGFR activity *in vivo*, and penetrates cells with its cargo [27, 28].

In the current study, we constructed a chimeric aptamer coupling E07 with a siRNA of *HOTAIR* in the same molecule and tested the chimera for its efficacy to deliver anti-*HOTAIR* RNA interference and suppression of cell growth and invasion in TNBC cells through EGFR.

Results

A synthetic DNA fragment containing the EGFR-targeting E07 aptamer, as reported by Li et al. [27], fused at the 3' end with the antisense sequence of a *HOTAIR* siRNA (*siHOTAIR*) was produced (Supplementary Figure 1). The synthesized DNA oligomers were then amplified by PCR with a T7 promoter sequence incorporated in the 5' primer followed by a T7-based *in vitro* transcription reaction. The RNA product of the EGFR aptamer coupled with *siHOTAIR*, hereafter termed *aptEGFR-siHOTAIR*. As a negative control, an aptamer in which the *siHOTAIR* sequence replaced with a control siRNA (*aptEGFR-siCtrl*) was generated.

We reasoned that the anti-EGFR aptamers should target cancer cells expressing EGFR while spare cells devoid of EGFR expression. Furthermore, the conjugated siRNA transported with the aptamer into the cells should downregulate the endogenous *HOTAIR* transcript. To test this hypothesis, we compared the breast cancer cell lines MDA-MB-231 and MDA-MB-468, both are EGFR-expressing cell lines, and MCF-7, which does not express EGFR. Analyses with confocal fluorescence microscopy of Cy3-labeled *aptEGFR-siHOTAIR* and *aptEGFR-siCtrl* showed that the aptamers were able to enter MDA-MB-231 and MDA-MB-468 cells but not

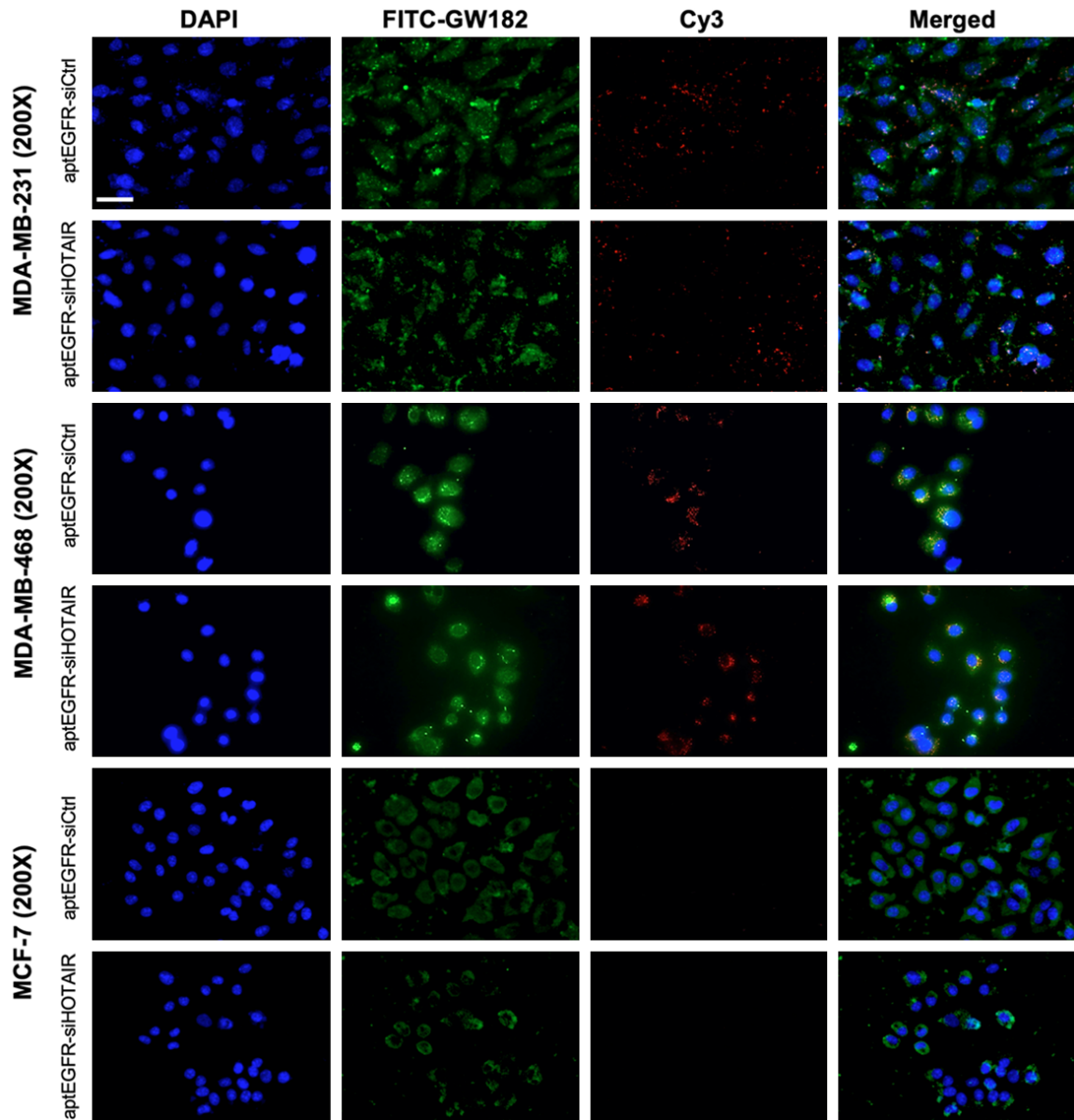


Figure 1. Cell entry of the aptamers to EGFR-positive cancer cells. MDA-MB-231, MDA-MB-468 and MCF-7 cells cultured in regular media were treated with Cy3-labeled *aptEGFR-siHOTAIR* or *aptEGFR-siCtrl* as indicated for 4 hr. Cells were then fixed and immunostained for GW182 (FITC). The nuclei were stained with DAPI. Magnification, 200 \times ; Scale bar, 50 μ m.

MCF-7 cells (**Figure 1**). The aptamer molecules in MDA-MB-231 and MDA-MB-468 cells formed punctates in the cytoplasm with partial co-localization with the multivesicular bodies (MVBs) as indicated by the endosomal marker GW182 [29], consistent with a cell-entry mechanism entailing membrane internalization. In drastic contrast, no aptamer was observed in MCF-7 cells. These results suggest that the EGFR aptamers targeted cancer cells through EGFR.

Both MDA-MB-231 and MCF-7 cells express endogenous the *HOTAIR* lncRNA. In line with its preferential internalization in MDA-MB-231 cells, *aptEGFR-siHOTAIR*, but not its negative control *aptEGFR-siCtrl*, significantly downregulated *HOTAIR* expression in MDA-MB-231 cells (**Figure 2A**). In contrast, *aptEGFR-siHOTAIR* had no effect in *HOTAIR* expression in MCF-7 cells (**Figure 2B**). These results predict that treatment with *aptEGFR-siHOTAIR* should have cancer-inhibitory activities in MDA-MB-231 but not

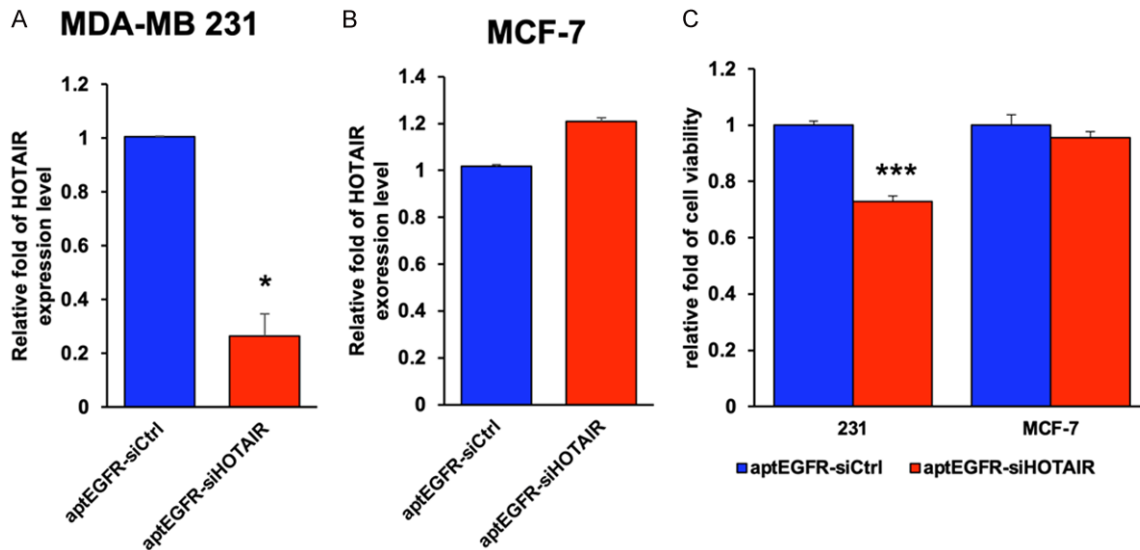


Figure 2. Depletion of the endogenous *HOTAIR* by the aptamer-delivered *siHOTAIR*. MDA-MB-231 and MCF-7 cells cultured in completed medium were transduced with the *aptEGFR-siHOTAIR* (*siHOTAIR*) or *aptEGFR-siCtrl* (*siCtrl*) for 72 hr. Cellular RNA of MDA-MB-231 (A) and MCF-7 (B) was extracted and the level of *HOTAIR* was measured by RT-PCR. Endogenous actin was used as the internal control. (C) Cell viability of MDA-MB-231 and MCF-7 cells treated with the indicated aptamer was assessed by crystal violet staining with the optical density (OD) measured by absorbance at 595 nm. Bar, standard deviation. *, $P < 0.05$; ***, $P < 0.005$.

MCF-7 cells. Indeed, compared to treatment with *aptEGFR-siCtrl*, *aptEGFR-siHOTAIR* treatment inhibited the viability of MDA-MB-231, but not MCF-7, cells (Figure 2C).

HOTAIR is well known for its function in enhancing migration and invasion of cancer cells [13, 15]. Administrating *aptEGFR-siHOTAIR*, but not *aptEGFR-siCtrl*, significantly suppressed the migratory ability of MDA-MB-231 (Figure 3A). More importantly, *aptEGFR-siHOTAIR*, but not *aptEGFR-siCtrl*, suppressed the invasive activity of MDA-MB-231 cells (Figure 3B), indicating that the strategy of the chimeric aptamer is an efficient strategy to suppress invasiveness mediated by *HOTAIR*.

To further test the dependence of EGFR in the biological function of the chimeric aptamer, MCF-7 cells were engineered to express ectopic EGFR or vector control alone (Figure 4A; Supplementary Figure 2). Expression of EGFR enhanced cell entry of the *aptEGFR* aptamer, with partial co-localization with the endosomal compartment under confocal microscopy analysis (Figure 4B). This was accompanied with reduced migratory activity of MCF-7 overexpressing EGFR, but not for the parental MCF-7 cells (Figure 4C). Importantly, introduction of EGFR enhanced invasive activity of MCF-7 cells

and treatment with *aptEGFR-siHOTAIR*, but not *aptEGFR-siCtrl*, significantly suppressed invasion mediated by EGFR expression (Figure 4D).

Discussion

Although EGFR is well known for its roles in driving cancer progression, metastasis, and resistance to treatments, targeting EGFR as single therapy has not made its way for approved breast cancer treatment due to unsatisfactory responsiveness of the patients. The observation that EGFR and c-ABL synergistically upregulate the oncogenic lncRNA *HOTAIR* provide an example that targeting downstream targets converging the signaling multiple pathways is a more promising strategy than targeting a single cancer driver. Alternatively, targeting key downstream targets dictated by the EGFR and c-ABL pathways can be an effective approach.

Our results showed that, compared to MCF-7 cells, MDA-MB-231 cells treated by *aptEGFR-siHOTAIR* preferentially downregulated *HOTAIR* expression with suppression of growth and invasion. The causal relationship of EGFR with the responsiveness is supported by ectopic expression of EGFR in MCF-7 cells which restored the cancer-inhibiting phenotypes by *aptEGFR-siHOTAIR* treatment. Loss-of-function

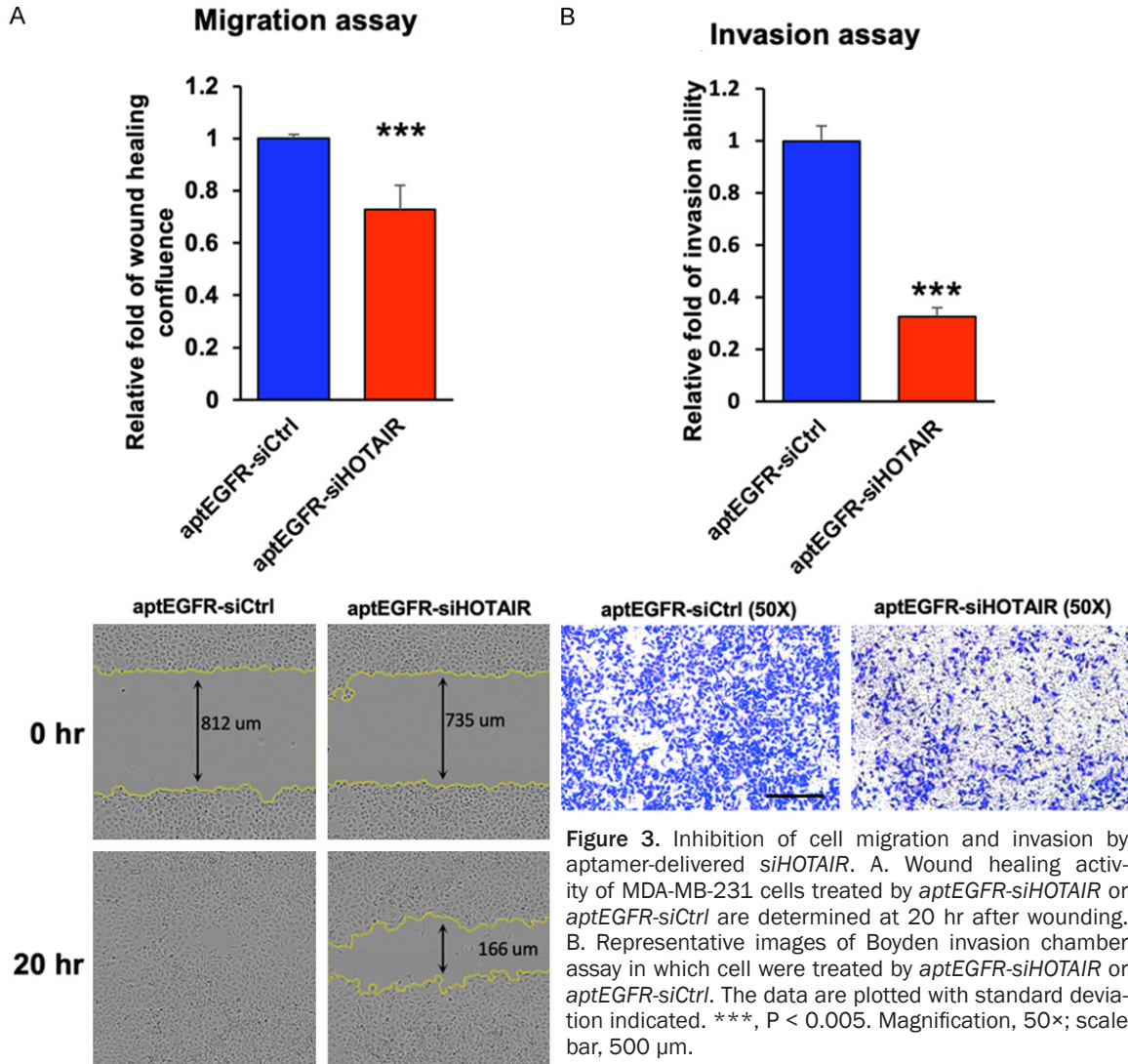


Figure 3. Inhibition of cell migration and invasion by aptamer-delivered *siHOTAIR*. A. Wound healing activity of MDA-MB-231 cells treated by *aptEGFR-siHOTAIR* or *aptEGFR-siCtrl* are determined at 20 hr after wounding. B. Representative images of Boyden invasion chamber assay in which cell were treated by *aptEGFR-siHOTAIR* or *aptEGFR-siCtrl*. The data are plotted with standard deviation indicated. ***, $P < 0.005$. Magnification, 50 \times ; scale bar, 500 μm .

approach by knocking down EGFR from DMA-MB-231 cells turned out to be non-feasible because of poor cell viability upon depleting EGFR in the cell line (data not shown). Overall, our finding is accountable for the scenario that the aptamer binds to the target EGFR and internalized through the endosome compartment with the cargo. It remains to be determined how the endosomal aptamer complex is released or gain access to the target RNA transcript in the cytosol. Our result showed that the internalized aptamers were colocalized with GW182, a protein enriched in the MVBs and the associated RNA-induced silencing complexes (RISCs) engaging in miRNA- and siRNA-mediated gene silencing [29].

The tumor-promoting functions of non-coding RNAs have been increasingly appreciated [30,

31], and the next hurdle to be overcome is the tumor-specific targeting of these biological moieties. To our knowledge this is the first report of delivering lncRNA-targeting RNA interference through aptamers targeting a receptor tyrosine kinase on cancer cells. Given that RTKs play important roles in tumor progression and metastasis, the concept proving principles demonstrated in the current study have substantial translational implication in cancer therapy and warrants further study with systemic administration of the aptamer in animal models for which an efficient and specialized delivery system is required [32]. Specifically the delivery system for the EGFR-targeting RNA aptamer should be able to present the EGFR-targeting moiety of the aptamer for tumor homing while protects the RNA cargos from nucleolytic attacks during circulation and allows efficient

RTK-specific aptamer-directed targeting of oncogenic long non-coding RNA

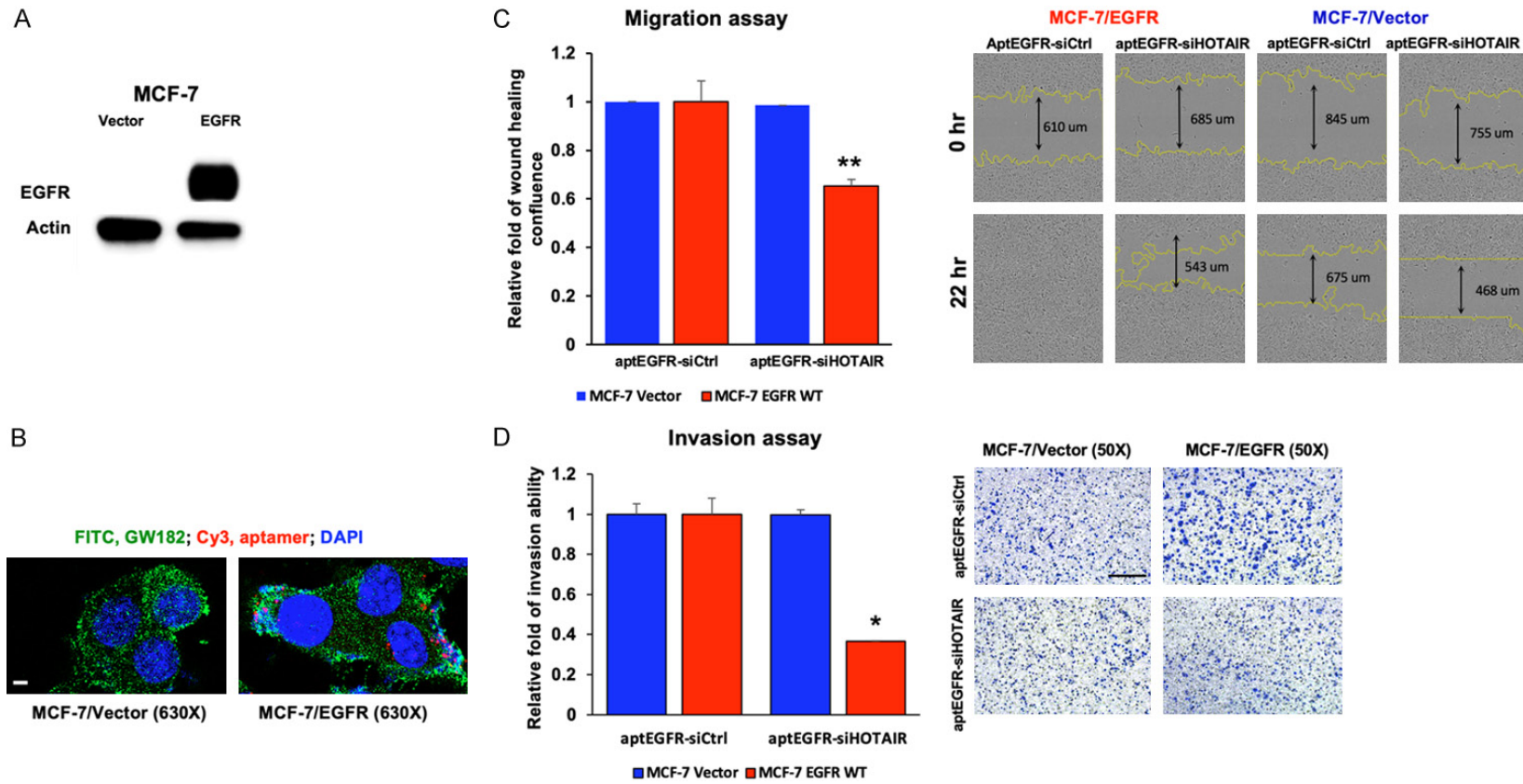


Figure 4. Inhibition of cell migration and invasion by aptamer-delivered *siHOTAIR* which specifically targeted to EGFR. (A) Ectopic EGFR expression in MCF-7. The original blots were cut according to the molecular weights of the proteins of interest. The resulted chemiluminescent images were cropped to keep only the relevant signals for space saving. (B) Confocal imaging of *aptEGFR-siHOTAIR* which was delivered in the presence of EGFR. Magnification, 50 \times ; scale bar, 5 μ m. Cell migration (C) and invasion (D) performed in ectopic EGFR expression of MCF-7 cells under treatment of *aptEGFR-siHOTAIR* or *aptEGFR-siCtrl* for 72 hr, followed by the corresponding assays for the indicated times. Representative images of Boyden invasion chamber assay in which cells were treated by *aptEGFR-siHOTAIR* or *aptEGFR-siCtrl* are shown. The data are plotted with standard deviation indicated. *, $P < 0.05$; **, $P < 0.01$. Magnification, 50 \times ; scale bar, 500 μ m.

RTK-specific aptamer-directed targeting of oncogenic long non-coding RNA

endosomal escape after entering the cells. Furthermore, the presentation of the double-stranded conformation of the aptamer in the cytosol may trigger immune response by the cytosolic nucleic acid-recognizing stress-response machinery, an important issue should be factored in to the assessment of the therapeutic outcome [33]. Overall, the current study provides a proof of the concept that aptamers homing on to the preys on the cell surface can be exploited for targeting intracellular non-coding tumor-promoting genes which are otherwise spared from the conventional therapeutics. Future study is warranted to test the *in vivo* efficacy in suppressing tumor progression and metastasis.

Methods

Cell lines, antibodies, and reagents

The human breast cancer cell lines: MCF-7 (ATCC®HTB-22™) and MDA-MB-231 (ATCC®HTB-26™) were obtained from the American Type Culture Collection (ATCC). The complete medium is DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), and 1% Pen-Strep human breast cancer cell lines. All cell lines were maintained at 37°C and 5% CO₂. Primary antibodies used in this research include the following: mouse monoclonal anti-GW182 (ab-70522, Abcam), rabbit polyclonal EGFR (ab-52894, Abcam), mouse monoclonal anti-Actin (sc32251, Santa Cruz Biotechnology). qRT-PCR primers of human genes: HOTAIR-forward, GG-TAGAAAAAGCAACCACGAAGC; HOTAIR-reverse, ACATAAACCTCTGTCTGTGAGTGCC; 18S-forward, AGGATCCATTGGAGGGCAAGT; 18S-reverse, TC-CAACTACGAGCTTTTAACTGCA; Actin-forward, CTTCCTCCATCGTGCGG; Actin-reverse, GTGGT-ACGGCCAGAGGCG; GAPDH-forward, CGGAGT-CAACGGATTTGGTTCGTA; GAPDH-reverse, AGCC-TTCTCCATGGTGGTGAAGAC.

Aptamer production

Aptamer is produced by *in vitro* transcription. Briefly, the *aptEGFR-siHOTAIR* template was amplified with the E07-F and E07-HOTAIR-R primers, with the T7 promoter (5'-GATAATAC-GACTCACTATA-3') incorporated in the E07 primer and the anti-sense sequence of the *siHOTAIR* siRNA (5'-GAACGGGAGTACAGAGAGA-3') in the E07-HOTAIR-R primer. The PCR product was then transcribed *in vitro* using the Platinum II

Taq DNA polymerase (Invitrogen) with which 1 µg PCR DNA template was estimated to produce about 100~150 µg of RNA product. The resulted RNA transcript contained the E07 *aptEGFR* aptamer linked with the anti-sense strand of the *siHOTAIR* siRNA. The RNA product was purified using the Directzol™ Kit (Zymo-research). The same approach was used to produce the control aptamer *aptEGFR-siCtrl* in which the siRNA was replaced with a scrambled sequence as the *siCtrl* (5'-GAGCGAGGGCGAC-TTAACCTTAGG-3'). The purified RNA (50 µM) was annealed with synthetic oligoribonucleotide of *siHOTAIR* or *siCtrl* by denaturing in annealing buffer (10 mM Tris, pH8.0, 1 mM EDTA and 50 mM NaCl) at 95°C for 5 min and let cool down to room temperature in the thermal cycler, -0.1°C/sec. For labeling, the RNA product was labeled with Cy3 by the *Label IT* Nucleic Acid Labeling kit (miR3600; Mirus).

The following oligonucleotides were used: *aptEGFR-siHOTAIR*-5'-GGCGCTCCGACCTTAGTCTCTGTGCCGCTATAATGCACGGATTAATCGCCGTAGAAAAGCATGTCAAAGCCGGAACCGTGTAGCACAGAGAAAAATCTCTGTACTCCGTTCTT-3' (the two underlined sequences are of *aptEGFR* and *siHOTAIR*, respectively); *aptEGFR-siCtrl*-5'-GGCGCTCCGACCTTAGTCTCTGTGCCGCTATAATGCACGGATTAATCGCCGTAGAAAAGCATGTCAAAGCCGGAACCGTGTAGCACAGAGAAAAAGAGCGAGGGCGACTTAACCTTAGGTT-3' (the two underlined sequences are of *aptEGFR* and *siCtrl*, respectively); E07-F-GATAATACGACTCACTATAGGCGCTCCGACCTTAGTCTCTG (the T7 promoter sequence is underlined); E07-HOTAIR-R-AAGAACGGGAGTACAGAGAGATTTTCTGCTGTGCTACACGGTTCCG (the two underlined sequences are of *siHOTAIR* and *aptEGFR*, respectively); E07-Ctrl-R-AACCTAAGGTTAAGTCGCCCTCGCTCTTTTCTGCTGTGCTACACGGTTCCG (the two underlined sequences are of *siCtrl* and *aptEGFR*, respectively).

HOTAIR quantitation

MDA-MB-231 and MCF-7 cells were plated in 6-well plates at a cell density of ~60%. Prior to aptamer treatment, cells were incubate in 500 µl of OPTI-MEM media at 37°C for 30 min, then treated with 400 nM of annealed aptamers in 500 µl of OPTI-MEM in the presence of 1 µl of RNaseOUT™ (Invitrogen), continue incubation for 1 hr, then added with 1170 µl of DMEM/F-12 with 14% FBS and incubated further for 72 hr.

RTK-specific aptamer-directed targeting of oncogenic long non-coding RNA

Cells were then lysed and the total RNA was extracted for quantitative RT-PCR (qRT-PCR). iQ™ SYBR® Green supermix (Bio-rad) was employed in qRT-PCR and a typical 2-step real-time PCR protocol was performed with an initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 sec and primer annealing and extension at 60°C for 30 sec.

Cell entry assay and endosome colocalization

MDA-MB-231 and MCF-7 cells were plated in 24-well plates (2.5×10^4 cells/well) for overnight. The cultured cells were washed and 300 μ l of OPTI-MEM media was added to each well and incubated for 30 min at 37°C. 50 nM of Cy3-labeled aptamers in 300 μ l of OPTI-MEM media was added and incubated at 37°C for 2 hr, followed by addition of 700 μ l DMEM media with 14% and further incubation for another 2 hr. Cells were fixed with pre-warmed 4% formaldehyde in 5% acetic acid for 30 min at room temperature, permeabilized in 0.25% Triton X-100/PBS for 10 min at temperature and then subjected to immunostaining with the GW182 antibody.

Wound healing assay

Wound healing assays was performed with two methods: a) Cells treated with either *aptEGFR-siCtrl* or *aptEGFR-siHOTAIR* were plated in 96-well plates (2×10^4 cells/well in MCF-7 while 1×10^4 cells/well in MDA-MB-231). The cell monolayer was scratched using a yellow pipette tip. The closure (migration distance) was assessed by photographing at 3, 6, 9, 12 and 24 hr, and measured by ImageJ. b) Cell migration was also measured by the IncuCyte ZOOM 96-well Scratch Migration assay protocol according to the manufacturer's instructions (Essen BioScience). A scratch wound was created in each well using the IncuCyte WoundMarker. Cells treated with either *aptEGFR-siCtrl* or *aptEGFR-siHOTAIR* were plated in 96-well plates. The wound healing process was monitored continuously in the IncuCyte Live-cell Imaging System. Images were captured every 2 hr for 24 hr.

Invasion assay

Cell invasion assays were performed in Transwell chamber inserts (Corning) which were

coated with a thin layer of Matrigel (Corning). 5×10^4 cells/well were suspended in serum-free medium and plated into each insert with the lower chambers filled with 500 μ l complete medium. After incubation for 24 hr or 48 hr, invading cells on the side of the Transwell facing the lower chamber were stained with crystal violet and quantitated by measuring the absorbance at OD 595 nm after solubilized in isopropanol.

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Disclosure of conflict of interest

None.

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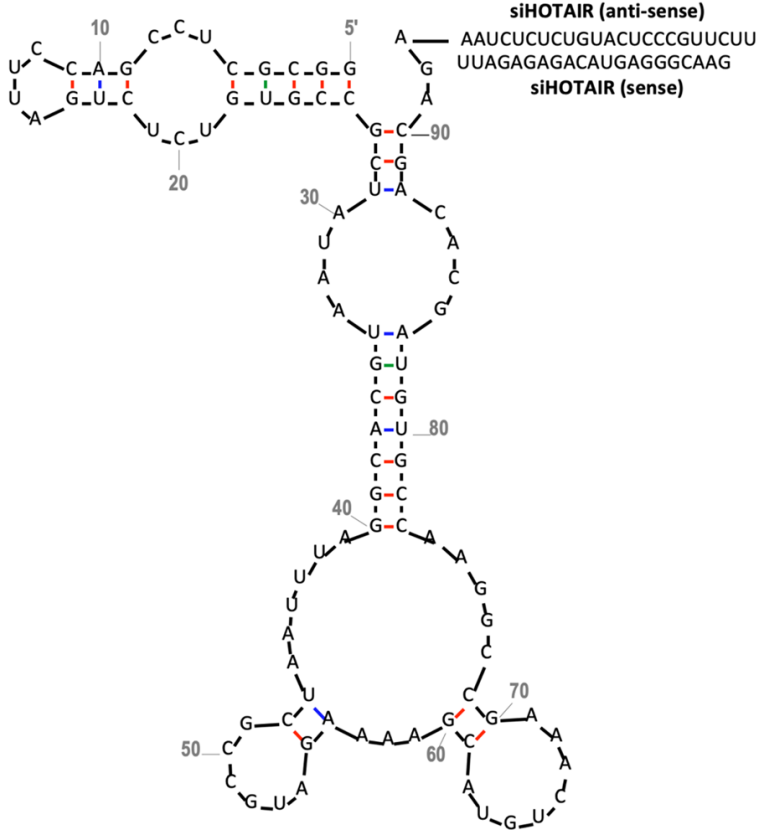
RTK-specific aptamer-directed targeting of oncogenic long non-coding RNA

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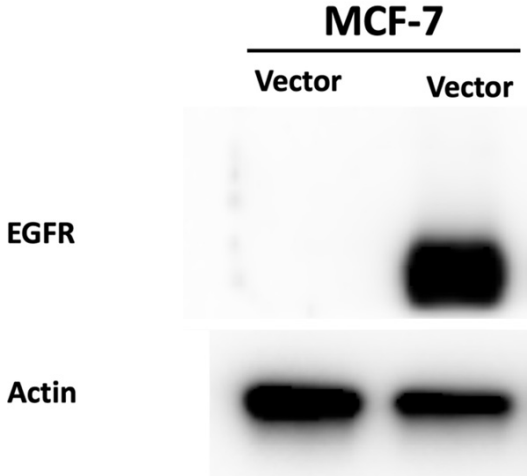
RTK-specific aptamer-directed targeting of oncogenic long non-coding RNA

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RTK-specific aptamer-directed targeting of oncogenic long non-coding RNA



Supplementary Figure 1. The tentative secondary conformation and full sequence of the *aptEGFR-shHOTAIR* aptamer. The aptamer is linked to the anti-sense strand of the siRNA at 3' end. The sense strand was subsequently annealed. The presented conformation, which is the same as reported by Li et al. [27], was created by the prediction program using The UNAFold Web Server (The RNA Institute College of Arts and Sciences, University at Albany, State University of New York).



Supplementary Figure 2. The original immunoblotting images for the result shown in **Figure 4A**. The whole blot was trimmed from 100 kDa and stained with the anti-EGFR antibody. To detect actin, the blot was cropped to leave the portion from 63 kDa to 35 kDa and incubation with the anti-actin antibody followed by chemiluminescent reaction for detection.