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Chaperone-mediated autophagy targeting chimeras (CMATAC) for the degradation of ERα in breast cancer

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Abstract: Estrogen receptor alpha (ERα/ESR1) is overexpressed in over half of all breast cancers and is considered a valuable therapeutic target in ERα positive breast cancer. Here, we designed a membrane-permeant Chaperone-mediated Autophagy Targeting Chimeras (CMATAC) peptide to knockdown endogenous ERα protein through chaperone-mediated autophagy. The peptide contains a cell membrane-penetrating peptide (TAT) that allows the peptide to by-pass the plasma membrane, an αI peptide as a protein-binding peptide (PBD) that binds specifically to ERα, and CMA-targeting peptide (CTM) that targeting chaperone-mediated autophagy. We validated that ERα targeting peptide was able to target and degrade ERα to reduce the viability of ERα positive breast cancer cells. Taken together, our studies provided a new method to reduce the level of intracellular ERα protein via CMATAC, and thus may provide a new strategy for the treatment of ERα positive breast cancer.

Introduction

Breast cancer (BC) is the most commonly occurring cancer in women (Bray *et al*., 2018). The majority of breast cancers are classified as ERα-positive BC, which is specified by the enhancement of the ERα signaling pathway in breast tumor tissue (Gajulapalli *et al*., 2016). The primary treatment for ERα-positive BC is hormonal therapy upon targeting ERα. Cite only directly pertinent references, and do not include data or conclusions from the work being reported. Tamoxifen, Fulvestrant, and aromatase inhibitors (AIs) comprise the hormonal therapy regimens for the treatment of ERα-positive breast cancer (Howell *et al*., 2004). However, drug resistance develops frequently in breast cancer patients, which is still a major obstacle for the efficacious treatment of breast cancer. Therefore, it is a significant and timely need to evolve new strategies to enhance hormonal therapy efficiency.

To explore a new therapeutic strategy, we have been attempted to harness the cellular protein degradation system to reduce levels of ERα protein. Here we took advantage of chaperone-mediated autophagy to decrease endogenous ERα by targeting it for lysosomal degradation. Mechanistically, our peptide system was able to efficiently reduce ERα protein by interacting with ERα and subsequent degradation via lysosomal in ERα-positive BC cells. We proposed that the utilization of chaperone-mediated autophagy may be translated into a novel therapeutic strategy for ERα-positive breast cancer.

Materials and Methods

Antibodies

Antibodies were as follows: anti-RFP (Abcam, ab62341, 1:1000 dilution), anti-ERα (CST, 8644, 1:1000 dilution), anti-GST (CST, 2624, 1:1000 dilution), anti-actin (Proteintech, 60008-1-Ig, 1:10000 dilution), anti-mouse secondary antibody, and anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology.

Cell culture

All cells (ATCC) were incubated in an atmosphere containing 5% CO_2 at 37°C, supplemented with 10% fatal bovine serum and 1% penicillin/streptomycin.

Plasmid construction

CTM-RFP and mCTM-RFP were constructed by introducing a fragment containing the CTM-RFP or mCTM-RFP coding sequence into the pEGFP-N2 vector. The cDNA for ERα, amplified by PCR from the first-strand cDNA of 293T cells, was cloned into the pEGFP-N2 vector. The TAT and αI

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coding sequences were prepared by annealing custom-designed oligonucleotides, and then ligated into pGEX-6P1 vector and CTM-RFP or mCTM-RFP plasmid.

Recombinant protein expression and purification

All the plasmid constructs were transformed into *Escherichia coli* BL21 (DE3), respectively. The transformed cells were cultured in Luria-Bertani (LB) broth at 37°C until OD600 of 0.6–1.0 and then induced with 0.1 mM IPTG. The expression of proteins was carried out at 25°C overnight. The purification step was performed, according to Shur *et al.* procedures (Shur *et al.*, 2013).

GST pull-down assay

GST pull-down assay was employed to identify the interactions between αI and ERα. In brief, GST-fused proteins were purified as previously described and incubated with prepared glutathione Sepharose beads on the rotating incubator at room temperature for 1 h, and then the beads were collected and washed three times. Cell lysates were added to these beads and subsequently rotated overnight at 4°C. After removing the supernatant, the beads were washed with the wash buffer three times. The target proteins were washed down with 10% SDS. These elutes were then analyzed and detected by SDS-PAGE and western blotting.

Peptide synthesis

Peptide was synthesized by Shanghai Dechi Bioscience Co., Ltd., following procedures described previously (Zhong *et al.*, 2019).

Western blot analysis

Proteins were extracted from cells using RIPA lysis buffer (Beyotime, China). Protein concentrations were determined with a BCA Protein Assay Kit (Beyotime, China). Equal amounts of protein per lane were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). Subsequently, membranes were blocked in 5% skim milk for 1 h and incubated overnight at 4°C with diluted antibodies. Finally, the membranes were incubated with HRP-conjugated secondary antibody (1:2500, Santa Cruz, USA). Blots were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce/Thermo Scientific, Rockford, IL).

Statistical analysis

The data are presented as mean ± SEM. Statistical analyses were performed on data collected from at least three independent experiments. Student's *t*-test (two-tailed) was used to compare two groups, and differences were considered statistically significant when $p < 0.05$.

Results

Knockdown of recombinant proteins

Chaperone-mediated autophagy (CMA) is a subtype of autophagy that delivers select proteins into the lysosome for degradation (Dice, 2007). CMA substrate proteins contain a peptide sequence biochemically related to KFERQ (Dice, 1990). The targeting peptide system is composed of three short peptides: A cell membrane-penetrating peptide (TAT) that allows the peptide to by-pass the plasma membrane, a protein-binding peptide (PBD) that specifically binds to the targeted protein, and CMA-targeting peptide (CTM) that activates the lysosome to degrade the targeted protein (Fan *et al.*, 2014).

According to a previous article, we first test whether our constructed CTM could direct the protein into the lysosome for degradation (Fig. 1A). In 293T cells, we transiently expressed the CTM-RFP construct, along with wild-type RFP as a control. Consistent with the ability of the CTM to direct CTM-RFP for lysosomal degradation, immunoblot analysis showed that the level of protein CTM-RFP was reduced more than mCTM-RFP (Fig. 1B). Consistently, CTM-GFP was predominantly colocalized with the lysosome marker protein LAMP1 (Fig. 1C). These data showed that a non-CMA substrate protein is able to degrade through the CMA pathway by the addition of CTM.

Properties of αI binding to ERα

ERα is a member of the steroid family of nuclear receptors that plays an important role in breast cancer initiation and progression (Alluri *et al.*, 2014; Heldring *et al.*, 2007). Most known proteins interact with ERα containing the LXXLL motif, and αI was designed as a probe that could specifically bind to ERα protein (Paige *et al.*, 1999). Therefore, we designed two αI constructs bearing GST tag, the TAT-αI with or without TAT-αI-CTM (Fig. 2A).

After purification on glutathione beads, the αI GST fusion proteins were incubated with cell lysates overexpressing full-length ERα. We found that the αI was associated with ERα tightly (Figs. 2B and 2C). Similarly, αI GST fusion proteins were purified and incubated with ERα-positive cell lysates, the result indicated that αI was able to interact with endogenous ERα (Fig. 2D). All these results suggested that αI was capable of binding to ERα.

TAT-αI-CTM inhibits ERα-positive breast cancer cell proliferation

The above results suggest that αI-CTM can knock down endogenous ERα to decrease the viability of cancer cells.

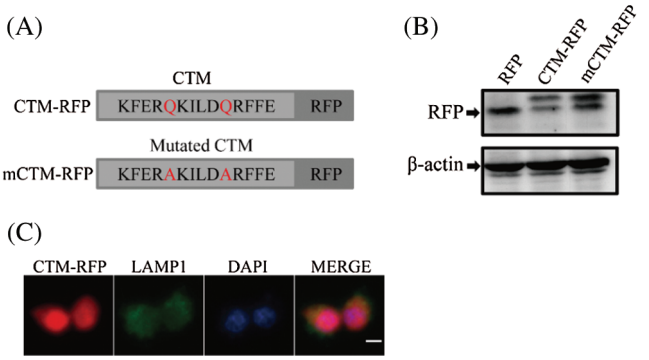

FIGURE 1. CTM-directed protein degradation.
(A) The architecture of CTM-RFP and mCTM-RFP. (B) Detection of CTM-RFP and mCTM-RFP and RFP protein level in HEK 293T cells after individual transfections with CTM-RFP, mCTM-RFP, or RFP alone. (C) Representative images of colocalization of CTM-RFP with the lysosome marker LAMP1 in MCF-7 cells transfected with CTM-GFP. Scale bar: 10 μm.

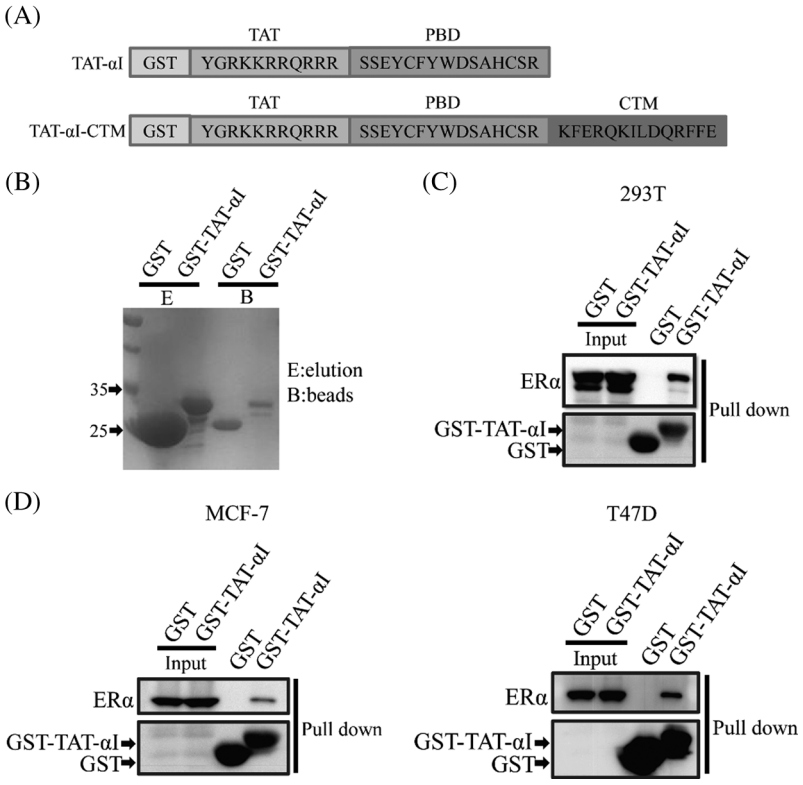

FIGURE 2. The binding of peptide αI to ERα.
(A) Design TAT-αI-CTM and TAT-αI peptides. (B) Production of GST and GST-TAT-αI using an *E. coli* expression system. Coomassie blue staining after SDS-PAGE assessed their purity. (C) Pull-down of TAT-αI and ERα. HEK 293T cells were transiently transfected with plasmids pEGFP-N2-ERα. 48 h after transfection, cell lysates were subjected to GST pull-down, and the pull-down fractions were immunoblotted analyzed. (D) Pull-down of TAT-αI and ERα in ERα-positive breast cancer cell lines, MCF-7 and T47D.

Next, we constructed and synthesized TAT-αI-CTM peptide by combining membrane-permeant targeting peptide TAT and αI-CTM (Fig. 3A). When applied with TAT-αI-CTM peptide, the level of ERα was decreased. And following increasing TAT-αI-CTM from 10 μg/mL to 200 μg/mL produced dose-dependent ERα degradation (Fig. 3B). Furthermore, we found that the application of TAT-αI-CTM decreased the viability of ERα-positive breast cancer cells and showed no toxicity on normal breast cells (Fig. 3C). These data strongly illustrate that ERα-targeting peptide TAT-αI-CTM can produce a reduction of ERα and inhibit the proliferation of ERα-positive breast cancer cells.

Together, these data provide valid evidence for the feasibility of our targeting peptide system. Furthermore, these results demonstrate the targeting peptide system can be used for knocking down native proteins in breast cancer

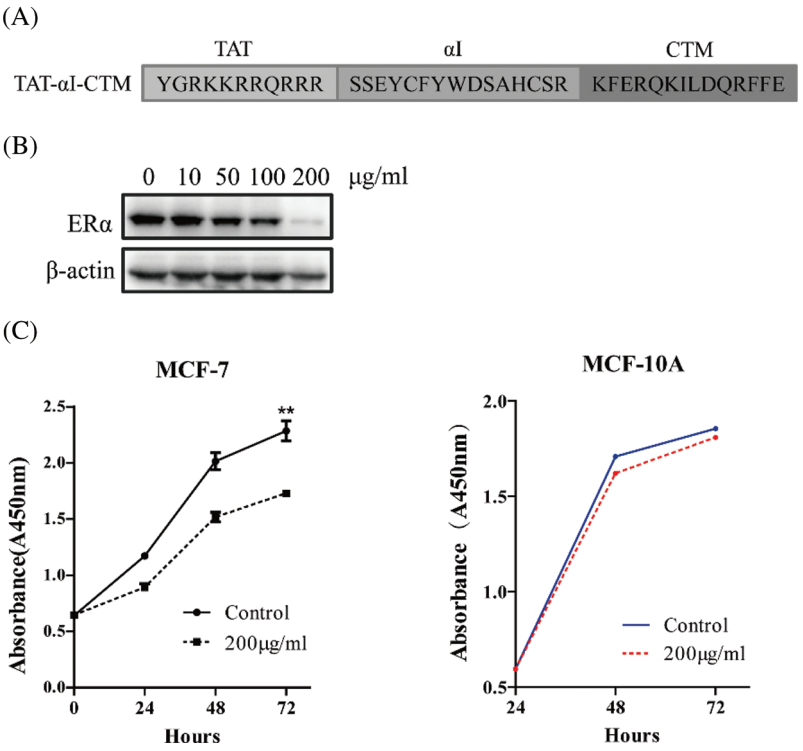

FIGURE 3. TAT-αI-CTM knocks down ERα, inhibiting the proliferation of ERα-positive breast cancer cells.
(A) Design and synthesis of TAT-αI-CTM peptide in Shanghai Dechi Bioscience Co, Ltd. (B) Incubation with TAT-αI-CTM peptide, knocked down ERα in a dose-dependent degradation. (C) Cell proliferation was measured using the CCK-8 kit after incubation with TAT-αI-CTM peptide (200 μg/mL), MCF-7, and MCF-10A cells were grown for 24, 48, and 72 h. The Student's *t*-test was used for statistical quantification: $^{**}p < 0.05$.

cells. Finally, our evidence suggests that targeting the peptide system may be suitable for use in designing therapeutic.

Discussion

In this study, we demonstrate a specific and efficient method to reduce the ERα protein level. Here we utilized ERα binding peptide αI to design a targeting peptide system and have proven the efficacy.

This method has several advantages compared to other methods, such as siRNA-mediated gene silencing. First, our system takes advantage of the cellular metabolism to replace outside interference, and cell damage is lower than other methods. Second, the targeting peptide system can be generated in several ways. It can be overexpressed by cDNA plasmid transfection, peptide synthesis and purified from bacterial expression systems. Therefore, it is a versatile system that can be widely used without sophisticated facilities.

Specificity is the most important property of the targeting peptide system. The effectiveness of targeting peptide in knocking down purpose proteins largely depends on the affinity and specificity of the interaction between the PBD and targeted proteins. The common methods to discover the appropriate PBD, including peptide arrays and phage display (Wu *et al.*, 2014); besides that, computational modeling can aid the discovery to increase the affinity and specificity. Therefore, the targeting peptide can be designed to play an anti-cancer role, which is specific for cancer cells by recognizing tumor markers.

This method can also be used as a complementary therapy for ERα-positive breast cancer to inhibit ERα action by the addition of TAT cell membrane-penetrating peptide. The TAT can deliver appointed cargo through the blood-brain barrier and plasma membrane into the interior of cells in various organs (Heitz *et al.*, 2009; Morris *et al.*, 2001; Tymianski, 2010). The efficacy and safety of TAT-mediated transduction of therapeutic peptides have recently been elucidated in a successful phase 2B clinical trials (Hill *et al.*, 2012). However, the stability of peptides has been problematic; recent developments in a range of modification techniques can be applied to enhance the stability of peptides. Thus, more efforts should be made to improve their therapeutic potentials.

In summary, our current study provides a peptide-based method that could efficiently knockdown endogenous proteins. It offers a new approach to regulate protein expression besides genetic and chemical means. We are convinced that targeting peptides offer enormous potential as an effective therapeutic method in the future.

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Conflicts of Interest: Nanjing Normal University has filed patent applications related to this work, listing L.C. and J.Z. as inventors.

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