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REGγ regulates ERα degradation via ubiquitin–proteasome pathway in breast cancer

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ABSTRACT

REGγ is a proteasome coactivator which regulates proteolytic activity in eukaryotic cells. Abundant lines of evidence have showed that REGγ is over expressed in a number of human carcinomas. However, its precise role in the pathogenesis of cancer is still unclear. In this study, by examining 200 human breast cancer specimens, we demonstrated that REGγ was highly expressed in breast cancers, and the expression of REGγ was positively correlated with breast cancer patient estrogen receptor alpha (ERα) status. Moreover, the expression of REGγ was found positively associated with poor clinical features and low survival rates in ERα positive breast cancer patients. Further cell culture studies using MCF7 and BT474 breast cancer cell lines showed that cell proliferation, motility, and invasion capacities were decreased significantly by REGγ knockdown. Lastly, we demonstrated that REGγ indirectly regulates the degradation of ERα protein via ubiquitin–proteasome pathway. In conclusion, our findings provide the evidence that REGγ expression was positively correlated with ERα status and poor clinical prognosis in ERα positive breast cancer patients. As well, we disclose a new connection between the two molecules that are both highly expressed in most breast cancer cases.

1. Introduction

REGγ, also known as Ki, PA28γ or PSME3, was first identified as Ki antigen, a nuclear protein targeted by autoantibodies found in the sera of patient with systemic lupus erythematosus [1]. REGγ is a member of 11S proteasome coactivator family [2,3]. It can stimulate the proteolytic activity of 20S proteasome and enable protein/peptide substrates to diffuse into the environment of proteasomes [4,5]. REGγ has been reported to be highly expressed in human breast cancers [6,7], thyroid cancers [8], hepatocellular carcinomas [9] and colorectal cancers [10]. In addition, recent studies have showed that REGγ play a role in protein degradation of tumor suppressor p53 [11,12], oncogene steroid receptor coactivator-3 (SRC-3) [13], as well as cell cycle regulator proteins including p21, p16 and p19 [14]. However, the precise role of REGγ playing in the tumorigenesis and development of human breast cancers is still unclear.

Breast cancer is the most frequently diagnosed cancer among women worldwide [15,16]. The majority (~70%) of breast cancers express high levels of estrogen receptor alpha (ERα) and so exhibit estrogen-dependent growth. The interaction between estrogen and ERα plays critical role throughout breast cancer development. Therefore, ERα, the regulation of which exhibits important values in breast cancer treatment, has been thought to be a key molecular target in breast cancer prognosis and treatment, as well as endocrine therapy. The expression of ERα is diverse in different breast cancers, and even in the same patient the expression level of ERα often changes at different stages of cancer development. The mechanisms through which ERα is regulated are complicated, including chromatin level [17], transcriptional level [18], post-transcriptional level [19] as well as post-translational level [20]. Recently, a few studies have reported the degradation of ERα is proteasome-dependent [21–25] although the detailed mechanism is still imprecise.

In the present study, we firstly examined the expression of REGγ in human breast cancers, and analyzed the relationship between REGγ expression and ERα status, as well as the prognosis in ERα positive breast cancer patients. Secondly, we investigated the changes in breast cancer cell proliferation, motility, and invasion capacity by REGγ knockdown. Finally, we explored the

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in vitro function of REGγ on regulating ERα protein expression, which involved the ubiquitin–proteasome mediated pathway. Taken together, we demonstrated that the expression of REGγ is positively correlated with patient ERα status and the poor clinicopathological features. Moreover, our data showed that REGγ may be involved in cancer development by indirectly regulating ERα protein degradation through ubiquitin–proteasome pathway.

2. Materials and methods

2.1. Patients and tissue samples

200 breast cancer with matching adjacent normal breast tissues (>3 cm) and axillary lymph node (ALN) tissues were obtained from patients who experienced modified radical mastectomy during 2008 and 2009 in breast disease center of Southwest Hospital. All cancer patients did not receive chemotherapy or radiotherapy before the surgery. All cases enrolled were followed up for 5 years. Written consent was obtained from each patient. This study was approved by the ethics committee of Southwest Hospital.

2.2. Cell culture

MCF7 and BT474 cells were obtained from American Type Culture Collection, USA. Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 mg/l penicillin and streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Reagents

REGγ monoclonal antibody (cat #710800), Lipofectamine 2000 (cat #11668-019), REGγ siRNA plasmid (sense sequence: gcaucuuaucuggaccagauuucua, antisense sequence: uagaaaucugguccagauaagaugc) and control (sense sequence: agcuacacuaucgagcaauuaacuu, antisense sequence: aaguuaauugcucgauaguguagcu) were purchased from Invitrogen, USA. Antibody against ERα (cat #sc-8002), β-actin (cat #sc-47778), and HA (cat #sc-805) were purchased from Santa Cruz Biotechnology, USA. Antibody against FLAG (cat #F1804) was purchased from Sigma–Aldrich, USA.

2.4. Immunohistochemistry

Immunostaining followed standard procedures. Anti-REGγ antibody (at 1:600 dilution), HRP-conjugated secondary antibody (cat #K4010, DAKO, USA) and DAB (cat #K3467, DAKO, USA) staining were performed as product reference. REGγ immunostaining scoring was based on the percentage of immuno-positive of staining within nuclei (<5% expression was regarded as negative, ⩾5% was regarded as positive). Scoring was evaluated by two authors in a blinded fashion, and an average of two scores was used to avoid inter-observer variability.

2.5. Western blot analysis

Samples were homogenized in RIPA buffer with proteinase inhibitors. Proteins were separated on SDS–PAGE, transferred to nitrocellulose membrane, and incubated with the corresponding antibodies after blocking. The blots were visualized by enhanced chemiluminescence method.

2.6. REGγ siRNA transfection

Cells were transfected with REGγ siRNA (CCUCGUUGCUGAAGG UGGAUCAGGA, UCCUGAUCCACCUUCAGCAACGAGG) or the control REGγ siRNA plasmid (AGCUACACUAUCGAGCAAUUAACUU, AAGUU AAUUGCUCGAUAGUGUAGCU) using Lipofectamine 2000 according to the manufacturer's instructions.

2.7. Cell proliferation, motility and invasion ability analysis

Cell proliferation was evaluated by MTS method (cat #G5421, Promega, USA). Cell motility was quantified using Cell Motility Kit (cat #K0800011, Thermo Scientific, USA). Cell invasion assay was performed using BioCoat Matrigel Invasion Chamber (cat #354480) and Falcon TC Companion Plate (cat #353504, BD Biosciences, USA) transwell kit, the invasion index was expressed as the ratio of the percent invasion of a test cell over the percent invasion of the control cell.

2.8. Quantitative PCR analysis

Total RNA was extracted with TRIzol (cat #T9424, Sigma–Aldrich, USA). Reverse Transcriptase Core kit (cat #RT-RTCK-05, Eurogentec, USA) was used to make cDNA libraries. StepOne Plus Real-Time PCR System (Applied Biosystems, USA) was used for qPCR. Primers and the corresponding TaqMan probes were designed in Roche Universal Probe Library Assay Design Center (Roche Applied Science, USA). House Keeping Gene 18S was employed as the internal control. The sequences of primers were as follows:

REGγ-F: 5′-acctaactcagatccactctgaca-3′, REGγ-R: 5′-tcatccaaccttcgcttctt-3′
ERα-F: 5′-ttactgaccaacctggcaga-3′, ERα-R: 5′-acctgatcatggagggtcaa-3′
18S-F: 5′-gtaacccgttgaaccccatt-3′, 18S-R: 5′-ccatccaatcggtagtagcg-3′

The delta threshold cycle value (ΔCt) was calculated using the formula ΔCt = Ct gene − Ct control. The fold change was calculated as $2^{-\Delta Ct}$.

2.9. ERα half-life chase and ERα-ubiquitin co-immunoprecipitation

For ERα half-life chase, cells were treated with 40 μg/ml cycloheximide (CHX) before harvesting. Proteasome inhibitor MG132 was used to eliminate proteasome degradation of ERα. For ERα-ubiquitin co-immunoprecipitation assay, cells were firstly transfected with exogenous expression plasmid carrying FLAG-tagged ERα and HA-tagged ubiquitin. After transfection, cells were incubated with 10 mM MG132 or vehicle reagent DMSO 6 h before harvesting. ERα was precipitated using anti-FLAG antibody at 1:500 dilution, the ubiquitinylation degradation of ERα was detected by anti-HA antibody at 1:500 dilution.

2.10. Statistical analysis

The correlation between REGγ expression and patients' clinicopathological parameters was studied using the chi-square test. Survival rates were estimated by Kaplan–Meier method and survival curves were compared using Log-rank (Mantel–Cox) test. GraphPad Prism 5.01 and IBM SPSS statistics 20 were used for statistical analyses.

3. Results

3.1. REGγ expression is positively correlated with ERα status and poor clinical features

Immunochemistry (IHC) staining data showed that REGγ was significantly highly expressed in breast cancer tissues (76%) and

metastatic ALNs (96.875%) compared to normal breast tissues (3%) ($p < 0.001$) (Supplementary Fig. 1 and Supplementary Table 1). Receiver Operating Characteristic (ROC) Curve analysis showed that REGγ positive expression have potential to distinguish the breast cancers from the normal breast tissues (Area = 0.865, Std. Error = 0.020, Asymptotic Sig. = 0.000, $p < 0.01$, 95% CI of Asymptotic is 0.826–0.904). We also examined the correlation of REGγ expression with cancer patient clinicopathological features, the detailed data was listed in Supplementary Table 2. As evaluated by IHC staining (Fig. 1A), the expression of REGγ was positively correlated with ERα status: high expression of REGγ was in accordance with positive status of ERα in patient with breast cancer. To further investigate the possible prognostic role of REGγ in the ERα positive breast cancer patients, we next performed survival analysis in ERα positive breast cancer patients. The Kaplan–Meier survival curves were plotted to display the 5-year survival rates of patients with REGγ-positive or REGγ-negative expression. We found that both overall survival (OS) and disease free survival (DFS) rate of patients in REGγ-positive group were significantly lower compared with REGγ-negative group (OS, $p = 0.0259$; DFS, $p < 0.0001$) (Fig. 1B and C). In ERα positive breast cancer patients, Cox proportional hazards regression analysis data indicated that REGγ could be a potential independent prognostic factor for breast cancer ($p = 0.027$, OR = 3.176, 95% CI of ratio is 1.282–9.808), and ERα positive status was a protective factor (OR = 0.108, 95% CI of ratio is 0.033–0.516) for breast cancer patients in this study. Collectively, our data confirmed that there is a significant positive correlation between REGγ expression and ERα status, as well as poor pathological features in ERα positive breast cancer patients.

3.2. Cell proliferation, mobility, and invasion ability is significantly decreased by REGγ knockdown

REGγ is highly expressed in many cancers including the breast cancer [6–10]. One possibility is that the high expression of REGγ is a bi-product and has little effect on cancer growth. However, REGγ has been found to promote the oncogenicity of MDA-MB-231 cells [6], combined with our findings that REGγ expression is positively correlated with poor pathological features of breast cancer, indicating that, instead of existing as a bi-product, REGγ plays a very important role in tumorigenesis and tumor development in breast cancer. We proposed that REGγ can promote tumor cell growth, invasion, and metastasis. To test this hypothesis, we investigated if REGγ knockdown by siRNA can affect cell growth, mobility, and invasion ability by running assays with two breast cancer cell lines MCF7 and BT474. Firstly we examined cell growth over 7 days for both cell lines with REGγ or control siRNA treatment. We found that, when REGγ expression was knocked down by REGγ siRNA treatment, cell growth declined significantly in both MCF7 ($p = 0.0033$) (Fig. 2A) and BT474 cells ($p = 0.0114$) (Fig. 2B). Next, we performed cell motility assay on both cell lines in order to examine if REGγ has an effect on cell mobility. Our data showed that the cell motility of both MCF7 ($p < 0.0001$) and BT474 cells ($p < 0.0001$) with REGγ siRNA treatment was significantly reduced

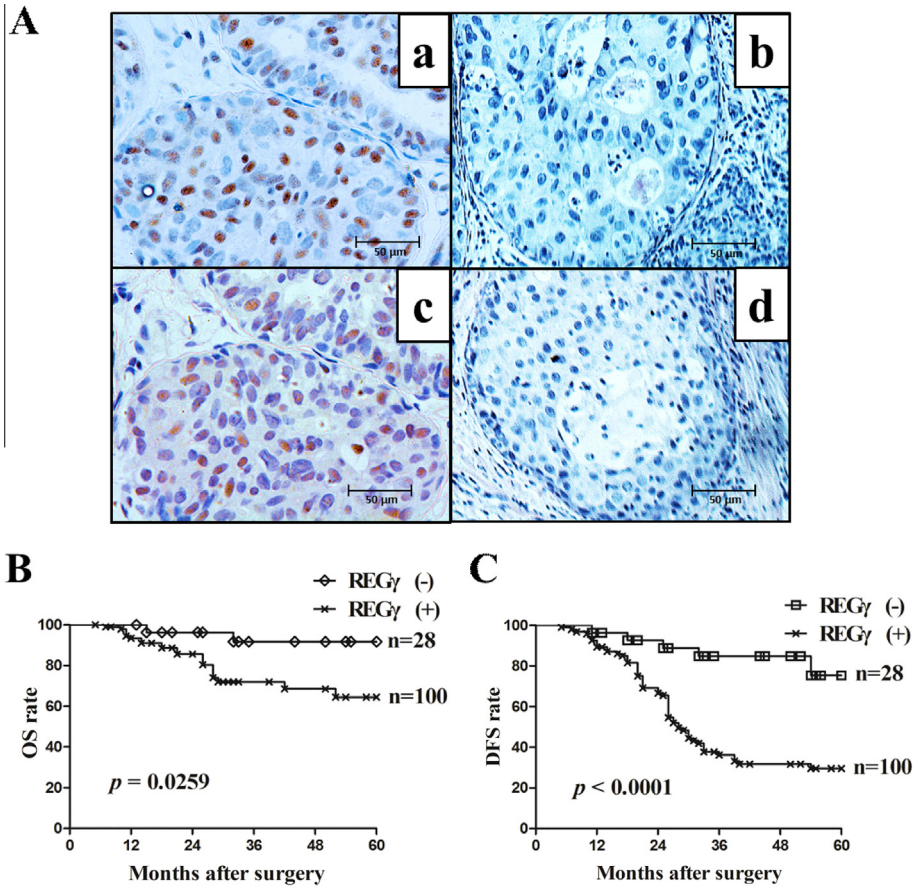

Fig. 1. REGγ expression is positively correlated with ERα status in human breast cancer. (A) Representative REGγ and ERα IHC staining images (bar = 50 μm). (a) ERα positive breast cancer tissue. (b) ERα negative breast cancer tissue. (c) REGγ highly expressed in ERα positive breast cancer tissue (contiguous slice with a). (d) No REGγ expression in ERα negative breast cancer tissue (contiguous slice with b). (B) Kaplan–Meier graph for OS time of REGγ negative and positive expression group in ERα positive breast cancers ($p = 0.0259$. Hazard Ratio = 0.3561, 95% CI of ratio is 0.1436–0.8831). (C) Kaplan–Meier graph for DFS time of REGγ negative and positive expression group in ERα positive breast cancers ($p < 0.0001$. Hazard Ratio = 0.3310, 95% CI of ratio is 0.1902–0.5758).

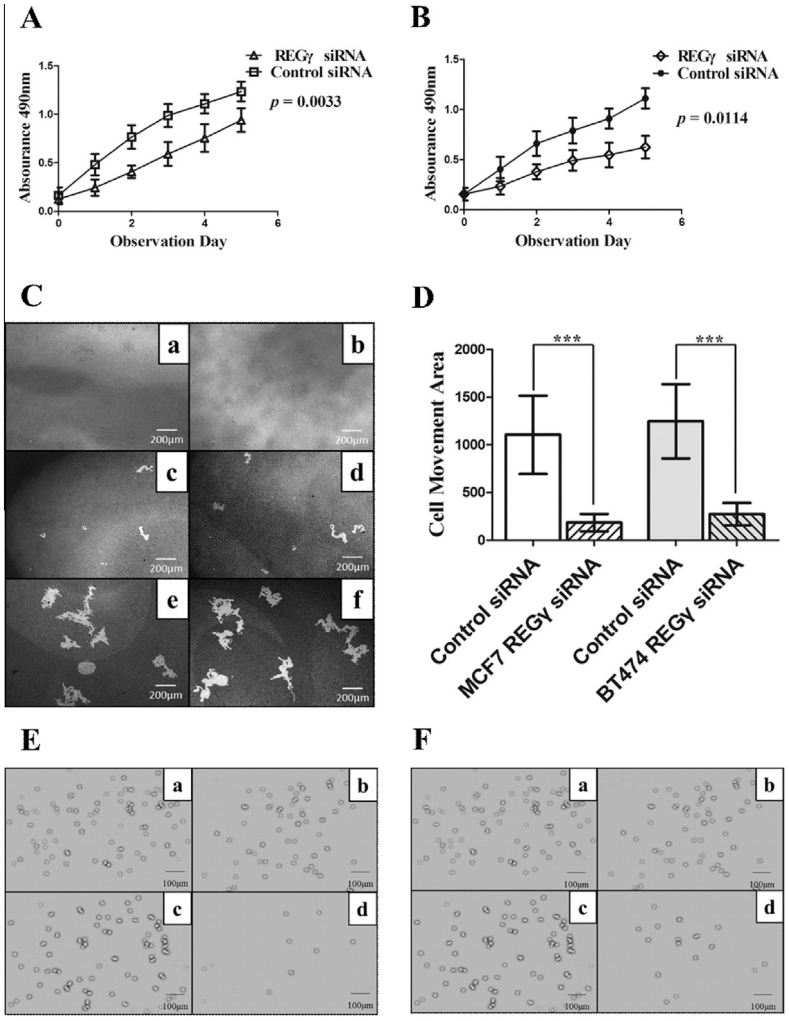

Fig. 2. Cell proliferation, motility, and invasion ability are impaired by REGγ knockdown. Proliferation curve showing that cell growth was decreased significantly by REGγ siRNA treatment in MCF7 ($p = 0.0033$) (A) and BT474 ($p = 0.0114$) (B) cells. (C) Representative cell motility assay images (bar = 200 μm). (a and b) Empty wells of 96-well plate coated with beads before seeding. Cell motility declined significantly by REGγ siRNA treatment in MCF7 (c) and BT474 (d) cells compared with the corresponding control cells (e and f). (D) Densitometric analysis showing cell motility measured by the movement trial area of different treated cells: the significant differences were found in both MCF7 ($p < 0.0001$) and BT474 ($p < 0.0001$) cells. Cell invasion assay results (bar = 100 μm): (a) control cells migrated through the matrigel matrix. (b) Control cells invaded through the matrigel matrix and membrane. (c) REGγ siRNA treated cells migrated through the matrigel matrix. (d) REGγ siRNA treated cells invaded through the matrigel matrix and membrane. The invasion ability decreased significantly by REGγ siRNA treatment in both MCF7 ($p = 0.0001$, invasion index = 0.3928) (E) and BT474 cells ($p = 0.0007$, invasion index = 0.2864) (F).

compared with cells treated with control siRNA (Fig. 2C and D). Last, we ran transwell assay to further assess the effect of REGγ knockdown on the invasion ability of cultured cancer cells. We found that the invasion ability of both MCF7 (Fig. 2E) and BT474 (Fig. 2F) was greatly reduced by REGγ siRNA treatment, compared with control siRNA treatment. The invasion index was 0.3928 and 0.2864, respectively. Taken together, our data suggested that REGγ may enhance breast cancer cell malignant progression by promoting cell proliferation, mobility, and invasion ability.

3.3. REGγ indirectly regulates ERα protein expression

REGγ is highly expressed in over 70% of 200 breast cancer cases we investigated. Similarly, ERα is over-expressed in about 70% of breast cancer cases. The regulation of ERα expression is complicated and involves many levels of modifications including polyubiquitination followed by proteasome-dependent degradation [21–25]. As a proteasome activator, REGγ plays a role in the degradation of many proteins including p53, SRC-3, p21, p16, etc. [11–14]. Based on this background, we proposed that REGγ is potentially involved in the regulation of ERα degradation and thereby promotes tumor growth and invasion. We employed ERα positive breast cancer cell lines MCF7 and BT474 to test this hypothesis. First, we treated cells with REGγ siRNA treatment and examined the changes in the protein and mRNA levels of ERα. We found that ERα protein expression levels decreased significantly by REGγ knockdown in both MCF7 ($p = 0.0053$) and BT474 ($p = 0.0035$) cells (Fig. 3A), while ERα mRNA levels had no significant change in both MCF7 ($p = 0.3011$) and BT474 ($p = 0.2178$) cells treated by REGγ siRNA (Fig. 3B), indicating that the regulation function of REGγ on ERα expression only took place on the post transcriptional level. Then we investigated the time course for the effect of REGγ knockdown on ERα expression by examining the ERα protein levels at different time points (0, 1, 2, 4 and 6 h) during treatment of siRNA. We found that ERα protein degraded more quickly in REGγ siRNA treated MCF7 ($p = 0.0093$) (Fig. 3C) and BT474 ($p = 0.0155$) cells (Fig. 3D), compared with control siRNA treated cells. Next, in order to

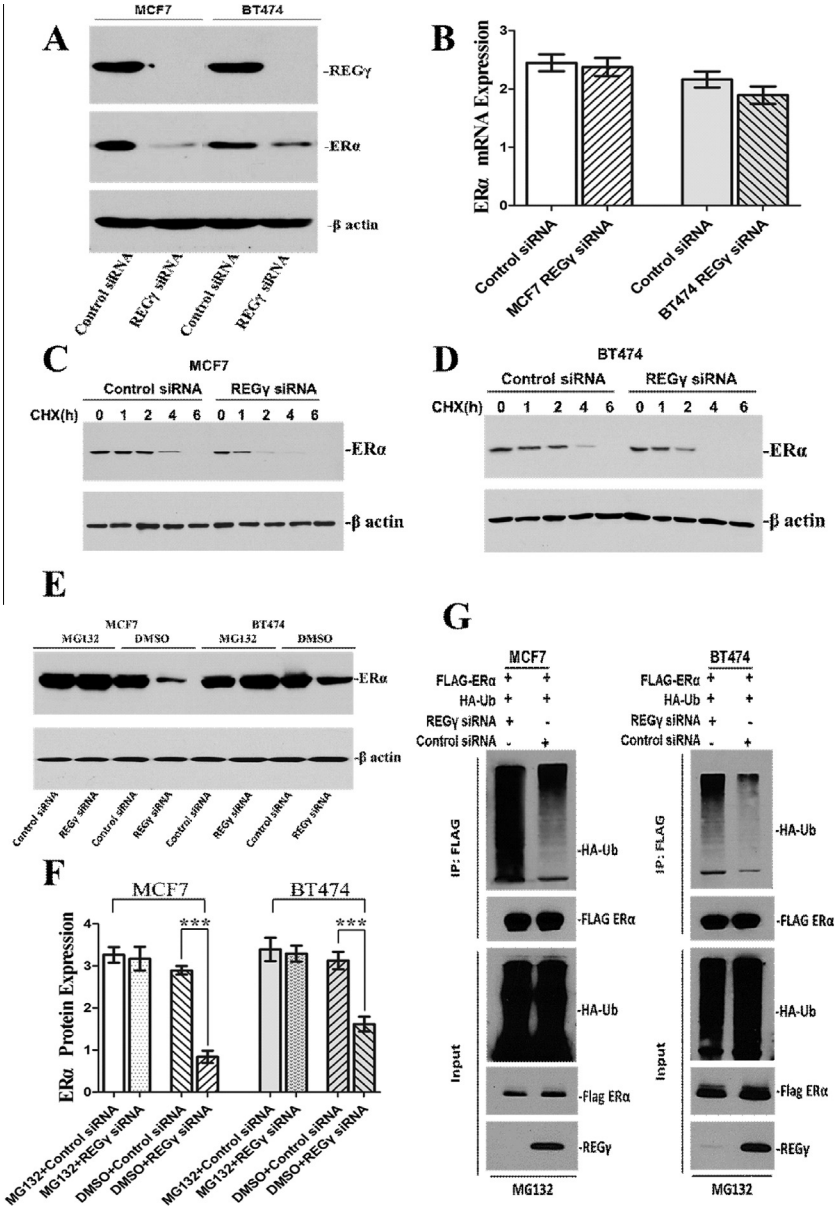

Fig. 3. REGγ regulates ERα protein expression via ubiquitin–proteasome mediated degradation. (A) Representative western blot of ERα protein expression after REGγ siRNA treatment: ERα was down-regulated in both MCF7 ($p = 0.0053$) and BT474 ($p = 0.0035$) cells. (B) No significant change of ERα mRNA expression was found in both MCF7 ($p = 0.3011$) and BT474 ($p = 0.2178$) cells after REGγ siRNA treatment. (C and D) ERα protein half-life time chase data showing that ERα degraded more quickly by REGγ siRNA treatment in MCF7 ($p = 0.0093$) (C) and BT474 cells ($p = 0.0155$) (D) compared with the control cells. (E and F) Western blot and densitometric analysis showing that MG132 abrogated ERα degradation caused by REGγ knockdown in MCF7 and BT474 cells. No significant difference of ERα expression was found in MG132 treated MCF7 ($p = 0.7642$) and BT474 cells ($p = 0.2440$) compared with control DMSO treated MCF7 ($p = 0.0002$) and BT474 cells ($p = 0.0003$). (G) Representative ERα-ubiquitin co-immunoprecipitation assay in MCF7 and BT474 cells showing that ERα was less polyubiquitinated with the presence of REGγ, indicating that REGγ may regulate ERα degradation indirectly.

investigate if the regulation of REGγ on ERα protein is dependent on the ubiquitin–proteasome pathway, we combined the treatment of proteasome inhibitor MG132 with siRNA treatment. We found that, not surprisingly, ERα degradation caused by REGγ knockdown was completely abrogated by proteasome inhibition (Fig. 3E and F), demonstrating that the regulation of ERα expression by REGγ is indeed mediated via proteasome-dependent pathway. Last, we performed ERα-ubiquitin co-immunoprecipitation to show that, in both types of cell lines, ERα protein was more heavily ubiquitinated when REGγ was knocked down, compared with control siRNA treatment (Fig. 3G), confirming that ubiquitin–proteasome pathway mediates the regulation of ERα expression by REGγ.

4. Discussion

Our finding that REGγ was highly expressed in breast cancer tissues and metastatic ALNs is in good accordance with previous studies [6,7]. We showed that REGγ was highly expressed in breast cancer tissues and metastatic ALNs whereas only 3% of normal breast tissues were REGγ positive, ROC Curve analysis also demonstrated that the expression of REGγ is distinguishable between malignant and normal breast tissues, which indicates that REGγ could be a potential diagnostic factor for breast cancer. Our study reveals that the high expression of REGγ was positively correlated with poor clinicopathological features for breast cancer, such as bigger tumor size, higher TNM stage, lower histological grade

and more number of metastatic ALNs. Moreover, in ERα positive breast cancers, REGγ positive patients exhibited lower DFS and OS rate compared with REGγ negative ones in 5-year follow up survival analyses. These phenomena implies a positive correlation between REGγ expression and breast cancer patient ERα status. Indeed, when we knocked down REGγ levels by siRNA in two breast cancer cell lines, cell proliferation, mobility, and invasion capacity was impaired. Therefore, REGγ may promote tumor growth by driving tumor cell proliferation and invasion. Our study helps to pave a new path for breast cancer therapy: REGγ inhibitors hold a high potential to be developed in the future as anticancer drugs.

The phenomenon that both REGγ and ERα are highly expressed in many breast cancer cases drives us to further investigate the potential association between REGγ and ERα expression. Previous studies have showed that ERα expression can be regulated via the post-translational modification such as polyubiquitination [21–25]. Several lines of evidence suggest that protein turnover and degradation, which is ultimately dependent on the ubiquitin–proteasome system [26–28], is the predominant mechanism for regulating cellular levels of ERα [29,30]. However, none of these studies focused on the relationship between proteasome regulators and ERα expression in breast cancers. Here for the first time we demonstrate that ERα expression is regulated by REGγ, a member of proteasome activator family. Although at first glance we would expect that as a proteasome activator, REGγ activates the degradation of ERα, which is actually not the case here, it is possible that REGγ regulates the degradation of an intermediate factor such as an E3 ubiquitin ligase that catalyzes polyubiquitination of ERα, followed by proteolysis of ERα by the proteasome. In another word, our data indicates that REGγ may regulate the degradation of ERα indirectly.

In conclusion, our study confirms the association between REGγ expression and ERα status in breast cancers, as well implied a potential target for future anti-cancer drug development. Most importantly, we address the novel regulation function of REGγ on ERα ubiquitin–proteasome mediated degradation, providing fresh ideas for better understanding the role of REGγ in tumor progression and also the regulation mechanism of ERα in breast cancer.

Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.124.

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