Published in final edited form as:

Breast Cancer Res Treat. 2012 June ; 133(2): 487–500. doi:10.1007/s10549-011-1775-9.

The rearranged during transfection/papillary thyroid carcinoma tyrosine kinase is an estrogen-dependent gene required for the growth of estrogen receptor positive breast cancer cells

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Abstract

The rearranged during transfection/papillary thyroid carcinoma (RET/PTC) tyrosine kinase is an oncogene implicated in the tumorigenesis of thyroid cancer. Recent studies by us and others have shown that RET/PTC kinase expression is induced by estrogen in breast cancer cells. Due to the critical involvement of estrogen-regulated genes in the pathogenesis of breast cancer, we investigated the expression, regulation and function of RET/PTC kinase in breast cancer cells. We found that RET/PTC kinase expression correlates with ER expression in breast cancer cells and tumor specimens, and that RET/PTC kinase expression is associated with a poor prognosis in ER-positive breast cancer patients. We found that estrogen rapidly induces RET/PTC kinase expression in an estrogen receptor (ER)-dependent manner in breast cancer cells and that this induction is through a transcriptional regulatory mechanism. Using reporter assays, small interfering RNA (siRNA) assays, and chromatin immunoprecipitation (ChIP) assays, we demonstrated the necessity of crosstalk between ER and the forkhead box A1 (FOXA1) transcription factor in regulating RET/PTC kinase expression. In functional studies, increased expression of RET/PTC kinase induced by estrogen stimulation resulted in elevated phosphorylation of multiple downstream kinase signaling pathways. Conversely, knockdown of RET/PTC expression was associated with the inhibition of these same kinase signaling pathways, and, in fact, decreased the stimulatory effect of estrogen on the proliferation of ER-positive breast cancer cells. These results demonstrate a novel pathway of ER and FOXA1 transcription factor crosstalk in regulating RET/PTC kinase expression, and demonstrate that RET/PTC kinase is a critical regulator for the proliferation of ER-positive breast cancer cells. Taken together, our study suggests that RET/PTC kinase may serve as a novel prognostic biomarker and therapeutic target for prevention and treatment of ER-positive breast cancer.

Keywords

estrogen; breast cancer; rearranged during transfection; papillary thyroid carcinoma; kinase; transcription factor crosstalk

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Part of this study has been presented in an abstract form at the AACR 102nd Annual Meeting, April 2011, Orlando, FL.

Conflict of interest

None declared.

Introduction

The estrogen signaling pathway is a critical regulator of the development and progression of breast cancer [1, 2]. The effects of estrogen on breast cancer are primarily mediated by estrogen receptor α (ER). In addition, ER can crosstalk with other transcription factors [3–6]. For example, we and others have shown that ER crosstalk with activating protein-1 (AP-1) [7], specificity protein 1 (Sp1) [8], and nuclear factor κ B (NF κ B) [9] to regulate target gene expression. Indeed, transcriptional profiling studies by us and others have identified many novel estrogen-regulated genes in breast cancer [10, 11], most of which are involved in cell growth regulation, cell cycle progression, and the suppression of apoptosis [10, 11]. Unfortunately, while a number of estrogen-regulated genes have been investigated in detail, the role of most of these genes play in the pathogenesis of breast cancer remains unclear.

In this study, we investigated the expression, regulation and function of a gene newly identified to be involved in breast cancer growth, the rearranged during transfection / papillary thyroid carcinoma (RET/PTC) tyrosine kinase. Previous studies have shown that RET/PTC kinase is a proto-oncogene highly expressed in neuroblastoma and papillary thyroid carcinoma [12, 13], and is associated with multiple endocrine neoplasia type 2A (MEN2A) syndrome [14]. Indeed, mutations and rearrangements of the RET/PTC kinase gene contribute substantially to the development of hereditary thyroid cancer [15, 16]. Furthermore, recent studies have shown that RET/PTC kinase signaling enhances estrogen-driven cell proliferation and enhances resistance to tamoxifen, an antagonist of ER in breast tissue and presently a standard endocrine therapy for ER-positive early breast cancer in premenopausal women [17, 18]. However, the specific function of the RET/PTC kinase gene in breast cancer has to date remained unclear.

In this study, we show that RET/PTC kinase expression correlates with ER expression in breast cancer cells and tumor specimens, as well as with poor prognosis in breast cancer patients. Using ER-positive cell lines as a model system, we have found that estrogen rapidly induces RET/PTC kinase expression in an ER-dependent manner by increasing the transcription of the RET/PTC kinase gene. We have also identified and demonstrated the necessity of ER-FOXA1 crosstalk in the regulation of RET/PTC kinase expression. In siRNA knockdown studies we demonstrated that the RET/PTC kinase is necessary for estrogen-induced growth. Furthermore, in functional studies we found that estrogen treatment induced expression of the RET/PTC kinase, which in turn augmented downstream kinase signaling pathways, and increased proliferation of breast cancer cells. This study demonstrates a novel pathway of estrogen regulation in the expression of this important kinase, and also provides unique insight into the specific therapeutic targeting of RET/PTC kinase in breast cancer treatment in the future.

Materials and Methods

Cells

BT474, HCC1428, HCC1500, HS578T, MCF-7, MDA-MB-175, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-453, MDA-MB-468, SKBr3, SUM-159PT, T47D and ZR75-1 breast cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). These cell lines were tested and authenticated using short tandem repeat (STR) profiling through MD Anderson's Institutional Characterized Cell Line Core. All cell lines were maintained in Improved Minimum Essential Medium (IMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). For estrogen treatment experiments, cells were switched to 10% charcoal-stripped fetal calf serum (Hyclone, Logan, UT) and cultured for at least 48h before estrogen treatment.

Tissue specimens and patient information

Clinical microarray datasets consisting of primary breast cancer tissue specimens were used to analyze the expression of the RET/PTC kinase gene. Raw data was obtained from the Oncomine database (<http://www.oncomine.com>). ER and RET/PTC kinase gene probe set IDs, the corresponding annotations, and the associated clinical data were extracted from the datasets. To evaluate the prognostic value of RET/PTC kinase gene expression in breast cancer patients, survival analysis was performed using the clinical microarray dataset with patient follow-up information. The RET/PTC kinase gene expression data was sorted according to its expression level, from highest to lowest, and categorized into three groups (high 1/3, medium 1/3, and low 1/3) based on RET/PTC kinase gene expression level. Kaplan-Meier analysis was performed using the overall survival data. In addition, the associated hazard ratio (HR) and 95% confidence interval (CI) was calculated.

Chemicals and treatment

The chemicals 17- β -estradiol (E2), ICI 182,780 (ICI), actinomycin D (ActD) and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). Glial cell line-derived neurotrophic factor (GDNF) was purchased from BD Biosciences and dissolved in PBS. The final concentrations of E2, ICI, ActD, CHX, and GDNF were 1nmol/l, 100nmol/l, 1 μ g/ml, 10 μ g/ml, and 1ng/ml, respectively.

Constructs

The promoterless pGL3basic plasmid (Promega, Madison, WI) served as the cloning backbone of all luciferase reporter plasmids. The plasmids containing RET/PTC kinase gene promoter fragments were provided by Dr. Ceccherini [19] and Dr. Garcia-Barcelo [20]. A series of five truncations, containing 5074bp, 2632bp, 1150bp, 184bp, and 84bp of the RET/PTC kinase gene promoter, were analyzed for estrogen responsiveness. The upstream RET/PTC kinase gene enhancer region (chromosome 10: 42841683-42842495bp) was cloned from human genomic DNA and inserted into the RET/PTC kinase promoter-luciferase plasmid. The primers are listed in Supplemental Table 1. A series of deletions and mutations were generated by site-directed mutagenesis. The ERE CCTCAGGGTGACC was mutated to CCTCAGGGTGctC. The ERE GGTGAGACTGGCT was mutated to GagGAGACTGGCT. The ERE half site TGACC was mutated to TGctC. Both FHRE sites TATTTGCTGA were mutated to TATaTGCTGA. All constructs were verified by sequencing.

Luciferase assay

Hormone-depleted MCF-7 cells were transfected with plasmids for 12h, then treated with vehicle or E2 for 6h, after which time they were lysed in Passive Lysis Buffer (Promega, Madison, WI). Luciferase activity was measured using a commercially available kit (Promega, Madison, WI). Each luciferase assay was performed in triplicate with confirmation of results established in two independent experiments.

RNA extraction and qRT-PCR

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed. Real-time quantitative PCR (qRT-PCR) was carried out on an ABI 7500 System (Applied Biosystems, Foster City, CA). The relative gene expression was determined using the comparative CT method and normalized to the housekeeping gene cyclophilin as described previously [21]. The primers and probes are listed in Supplemental Table 1. Each qRT-PCR assay was performed in triplicate and results were confirmed in dual independent experiments.

siRNA experiments

Hormone depleted cells were seeded into 6-well plates, followed by transfection of the cells with chemically synthesized siRNAs (Sigma-Aldrich Corporation, St. Louis, MO) using RNAiMAX reagent (Invitrogen, Carlsbad, CA).

Western blotting procedure

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Roche, Basel, Switzerland). The proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After incubating sequentially with primary and secondary antibodies, the blots were developed using SuperSignal West Pico kit (Pierce, Rockford, IL) and exposed to film. All antibodies are listed in Supplemental Table 2. Each Western blot experiment was performed in triplicate and a representative result is shown.

Chromatin immunoprecipitation (ChIP) procedure

ChIP experiments were performed according to published protocols with minor modifications [22, 23]. Hormone depleted MCF-7 cells were grown in 15cm dishes. After treatment with vehicle or E2 for 30 min, cells were washed twice with PBS and cross-linked with 1% formaldehyde in PBS for 10min. The cross-linking reaction was stopped by washing with PBS containing glycine (final concentration 0.1mol/l), and the cells were then scraped and lysed. Subsequently, sonicated chromatin was immunoprecipitated with antibodies as indicated, followed by purification of the immunoprecipitated fragments using QiaQuick columns (Qiagen, Valencia, CA). These fragments were then used as templates for q-PCR. The primers and probes are listed in Supplemental Table 1. The recruitment was presented as the ratio of q-PCR result of immunoprecipitated fragments normalized to input. Each q-PCR assay was performed in triplicate and confirmation of results was established by dual independent experiments.

Cell proliferation assays

Cell proliferation was determined through both 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays and anchorage independent soft agar colony formation assays.

For MTS assays, cells were grown in 96 well plates. After the indicated number of days, MTS assays were performed using the CellTiter 96 kit (Promega, Madison, WI) according to the manufacturer's instructions. The optical density at 490 nm was read with an iMark microplate reader (Bio-Rad, Hercules, CA). All MTS assays were performed in triplicate with the results confirmed in two independent experiments.

For soft agar colony formation assays, IMEM containing 10% CSS and 0.7% Sea Plaque agar was solidified in 6-well plates to form the base agar layers. For top agar layers, 5000 cells were mixed with IMEM containing 10% CSS and 0.35% Sea Plaque agar and added onto the base agar layers. After solidification at room temperature, cells were grown for 2~4 weeks with fresh media added every 3 days. Colony formation was monitored under microscopy. Colony number was determined using GelCount machine and software (Oxford Optronix, Abingdon, United Kingdom). All soft agar colony formation assays were performed in triplicate with confirmation of results established by dual independent experiments.

Results

Expression of RET/PTC kinase in breast cancer cells and tumor tissue specimens

To gain insight into the role of RET/PTC kinase in breast cancer, we examined its expression in a panel of breast cancer cell lines. As shown in Figure 1A and 1B, RET/PTC kinase mRNA and protein are differentially expressed throughout these cell lines, with mRNA level in accordance with protein level in most of the cell lines. Of particular interest, expression of RET/PTC kinase is significantly higher in ER-positive cell lines in comparison to ER-negative cell lines ($P=0.0004$, Figure 1C). In fact, qRT-PCR analysis revealed undetectable RET/PTC mRNA levels in most ER-negative cell lines. Similar results were obtained through cDNA microarray analysis of a dataset comprised of 51 breast cancer cell lines reported by Neve *et al* [24] ($P<0.0001$, Supplemental Figure 1A). Upon examination of the clinical microarray datasets, RET/PTC kinase mRNA was found to be significantly overexpressed in ER-positive versus ER-negative breast tumor tissue specimens in all analyzed independent datasets, collectively representing tissues from over 2,000 patients (Oncomine database). Of these independent datasets, the three largest (each consisting of more than 190 patients), with their respective RET/PTC kinase mRNA expressions, are depicted in Figure 1D [Bittner *et al*/International Genomics Consortium (IGC), Not Published, 2005; Desmedt *et al*/Translational Breast International Group (TRANSBIG) [25]; Wang *et al*/Veridex LLC (Veridex) [26]]. We also identified a significant correlation between RET/PTC kinase expression and ER expression in breast tumor tissue specimens from all the datasets. This analysis showed that breast tumors with high ER expression have high RET/PTC kinase expression [Figure 1E ($P<0.0001$) [25] and Supplemental Figure 1B].

In order to more clearly define the potential prognostic value of RET/PTC kinase expression in breast cancer, we performed a survival analysis using the information provided for the ER-positive breast cancer patients from the clinical microarray dataset with follow-up information [25]. Kaplan-Meier analysis defined a significantly worse prognosis for those individuals with tumors expressing higher levels of RET/PTC kinase than for those associated with lower levels of RET/PTC kinase expression, as evidenced by a significantly lower overall survival percentage rate (Hazard ratio=3.38, 95% confidence interval: 1.37–8.33, Mantel-Cox log-rank test $P=0.0081$) (Figure 1F).

RET/PTC kinase is an estrogen-regulated gene

Since RET/PTC kinase is an ER-associated gene, we tested the effect of estrogen on its expression in a panel of breast cancer cell lines. As shown in Figure 2A, 17- β -estradiol (E2) induced RET/PTC kinase mRNA expression in all ER-positive breast cancer cell lines analyzed, but had no effect in MDA-MB-453, the only ER-negative breast cancer cell line with detectable RET/PTC kinase mRNA expression in the analysis. To investigate the underlying molecular mechanism by which estrogen regulates RET/PTC kinase expression, we chose the ER-positive MCF-7 breast cancer cell line as a model system for mechanistic studies, as it displayed the strongest E2-regulated induction of RET/PTC kinase expression. We next defined the time course of the expression levels of RET/PTC kinase mRNA in response to estrogen treatment. As shown in Figure 2B, estrogen induces RET/PTC kinase mRNA expression robustly, with induction prominent as early as 1h after E2 treatment, with high levels remaining for at least 6h. Expression of RET/PTC kinase protein was confirmed through Western blot analysis showing E2 induction of RET/PTC kinase protein expression in MCF-7 cells after 24h of E2 treatment (Figure 2B, inset). This rapid induction suggests a mechanism of transcriptional regulation for estrogen-mediated regulation RET/PTC kinase expression. In order to confirm that RET/PTC kinase is regulated at the transcriptional level as opposed to translational or post-translational regulation, we tested the effect of inhibitors

of both RNA synthesis, actinomycin D (ActD), and protein synthesis, cycloheximide (CHX), versus that of a vehicle, dimethyl sulfoxide (DMSO) on E2-induced RET/PTC kinase expression. As shown in Figure 2C, ActD, but not CHX, was able to block E2-regulated induction of RET/PTC kinase mRNA expression, indicating that E2 induction of RET/PTC kinase gene is dependent upon RNA transcription but not new protein synthesis. Therefore, estrogen-mediated regulation of RET/PTC kinase expression is via a primary transcriptional regulatory mechanism.

To determine whether estrogen-induction of RET/PTC kinase expression requires ER, we investigated the effect of estrogen after pretreatment with pure ER antagonist, ICI 182,780 (ICI), or with ER siRNA. Loss of expression of ER protein was confirmed through Western blot analysis (Figure 2E: Inset). As shown in Figure 2D and 2E, both ICI and ER siRNA independently and completely blocked E2-induced expression of RET/PTC kinase. These results show that estrogen induction of RET/PTC kinase depends on ER.

RET/PTC kinase gene expression is regulated by estrogen through an upstream enhancer

Since RET/PTC kinase is an ER/E2-dependent gene, to further characterize the mechanics of this interaction, we investigated whether the proximal promoter of RET/PTC kinase plays a role in the estrogen inducibility of this gene. Previous studies have shown that the RET/PTC kinase promoter can be transactivated by Sp1 [19, 27], which may tether ER to the promoter by transcription factor crosstalk. We tested five differentially truncated lengths of RET/PTC kinase promoter fragments, ranging from –84bp to –5074bp, and identified none that were responsive to estrogen treatment (Figure 3A). Previously, we had observed that binding of ER to distal enhancer regions was sufficient to allow for the regulation of *c-myc* gene expression [7], therefore we tested whether RET/PTC kinase gene is also activated by a distant enhancer. By examining multiple databases of ER binding sites [28–35], we discovered an ER binding region (813bp in length, specifically 42841683–42842495bp of chromosome 10) located approximately 50kb upstream of the RET/PTC kinase gene locus (Figure 3B). This region was identified by eight independent studies, supporting the hypothesis that it may serve as a functional enhancer contributing to the estrogen-induction of RET/PTC kinase expression. We therefore cloned this 813bp fragment upstream of the proximal promoter (a core promoter with strong basal activity lying –184bp~+190 of the transcriptional start site). As shown by the increase in luciferase activity upon treatment with estrogen (Figure 3C), this distal DNA fragment is highly responsive to estrogen, thereby indicating that it is capable of functioning as a true enhancer.

RET/PTC kinase gene expression is regulated by ER-FOXA1 crosstalk

To further evaluate which *cis*-acting elements and cognate *trans*-acting factors are required for estrogen-regulated induction of RET/PTC kinase expression, we analyzed the 813bp enhancer region for the presence of transcription factor response elements. Five potential transcription factor binding sites were identified, including two forkhead responsive elements (FHREs), two estrogen responsive elements (EREs), and one ERE half site (hERE). The locations and sequences of these transcription factor binding sites are depicted in Figure 4A. To determine the potential estrogen inducibility associated with each of these transcription factor binding sites, we performed site-directed mutagenesis of each of the five binding sites and tested whether a single mutation within any of these sites was capable of significantly altering the estrogen-inducible activity of the reporter construct. As shown in Figure 4B, mutations within the first FHRE (FHRE#1), second ERE (ERE#2), and the hERE had little effect on estrogen-induced luciferase reporter activity, indicating that these sites are not physiologically relevant to estrogen regulation of RET/PTC kinase gene expression. By contrast, mutation of either the second FHRE (FHRE#2) or the first ERE (ERE#1) significantly inhibited estrogen-induced luciferase reporter activity, indicating that both of

these sites are involved in estrogen induction of RET/PTC kinase gene expression. To assess the possibility of a cooperative effect between these two binding sites, we then performed a double mutation of FHRE#2 and ERE#1. The double mutation affecting both of these elements abolished estrogen-induced reporter activity, indicating that these two transcription factor binding sites together comprise the critical regulatory region for estrogen induction of RET/PTC kinase expression (Figure 4B). Interestingly, these two sites are positioned adjacently to one other (lying only 88bp apart). This suggests a cooperative association between the transcription factor binding sites for ER (ERE#1) and forkhead A1 (FOXA1) (FHRE#2) with respect to the regulation of RET/PTC kinase gene expression.

To investigate the involvement FOXA1 in estrogen regulation of RET/PTC kinase gene expression, we performed both knockdown and overexpression experiments. As shown in Figure 4C, knockdown of FOXA1 significantly reduced basal level and estrogen-regulated RET/PTC kinase expression, demonstrating that FOXA1 is necessary for basal and induced RET/PTC kinase expression. Overexpression of FOXA1 increased estrogen-regulated RET/PTC kinase expression slightly as shown in Figure 4D. This modest increase suggests that endogenous FOXA1 is sufficient to facilitate estrogen-regulated induction of RET/PTC kinase gene expression. To ensure that FOXA1 knockdown or overexpression did not affect ER expression, we performed a Western blot for ER and found that ER protein expression levels were not influenced by either the knockdown or overexpression of FOXA1 (Figure 4CD: Insets).

ER and FOXA1 proteins are recruited to the upstream enhancer

We next performed ChIP analysis to verify the recruitment of ER and FOXA1 proteins to the regulatory regions (ERE#1 and FHRE#2) under physiological conditions. Immunoprecipitated DNA was analyzed by q-PCR using primers for both the enhancer and proximal promoter regions (Figure 5A). As shown in Figure 5B and 5C, ER and FOXA1 proteins, respectively, were recruited to the enhancer but not to the promoter regions when the cells were treated with estrogen. FOXA1 proteins were recruited to the enhancer region even in the absence of estrogen, indicating a potential pioneering role for the FOXA1 transcription factor in ER-mediated regulation of RET/PTC kinase expression [31]. RNA polymerase II (PolII), which is required for mRNA transcription, was recruited solely to the RET/PTC kinase proximal promoter in response to estrogen (Figure 5D), serving as further confirmation that estrogen induction of RET/PTC kinase gene expression is via a primary transcriptional regulatory mechanism.

RET/PTC kinase signals through multiple kinase pathways to regulate cell proliferation

Since we demonstrated that RET/PTC kinase is an ER-dependent tyrosine kinase, we next sought to determine whether regulation of its expression by estrogen contributes to the downstream regulation of kinase signal transduction pathways. As shown by Western blot analysis in Figure 6 estrogen treatment resulted in upregulation of RET/PTC kinase expression and augmented levels of phosphorylated RET/PTC kinase (p-RET), phosphorylated 44kD/42kD mitogen-activated protein kinase 1 / extracellular signal-regulated kinase (p-ERK), phosphorylated v-akt murine thymoma viral oncogene homolog 1 (p-AKT), and phosphorylated 38kD mitogen-activated protein kinase 14 (p-p38-MAPK), while not upregulating unphosphorylated ERK, AKT or p38-MAPK. Conversely, knockdown of RET/PTC expression was associated with the inhibition of these same kinase signaling pathways. Collectively, these data supports the idea that RET/PTC kinase participates in the regulation of kinase signal transduction pathways. Furthermore, since these kinases (ERK, AKT and p38-MAPK) are all critical regulators of cell proliferation, these results led us to ask whether RET/PTC kinase regulates estrogen-induced growth. Therefore, we next examined the role of RET/PTC kinase in regulating breast cancer cell

growth. As shown in Figure 7A, estrogen significantly stimulated the proliferation of ER-positive MCF-7 cells, while knockdown of the RET/PTC kinase via siRNA downregulated both estrogen-induced and, to a much lesser extent, basal MCF-7 cell proliferation. By contrast, neither estrogen nor siRNA inhibition of RET/PTC kinase had any effect on the proliferation of ER-negative MDA-MB-453 breast cancer cells (Figure 7B), which is the only ER-negative cell line that expresses the RET/PTC kinase. We next performed soft agar colony formation assays to assess cell proliferation in three-dimensional anchorage-independent culture conditions. Once again, as shown in Figure 7C (Upper Panel), estrogen significantly stimulated colony formation of the ER-positive MCF-7 cells, whereas knockdown of RET/PTC kinase inhibited colony formation of these cells. Colony counts are depicted in Figure 7C (Lower Panel). To confirm the involvement of RET/PTC kinase in ER-positive breast cancer cell proliferation, we repeated these experiments using an additional ER-positive cell line (T47D) and obtained similar results (Supplemental Figure 2). Together, these results demonstrate that RET/PTC kinase is a critical regulator for proliferation of ER-positive breast cancer cells.

Discussion

In the present study, we investigated the expression, regulation, and function of RET/PTC kinase, an oncogene previously implicated in tumorigenesis of thyroid cancer, in breast cancer. We found that RET/PTC kinase is overexpressed in ER-positive breast cancer cell lines and breast tumor specimens, and that high expression of RET/PTC kinase is associated with a poor prognosis. We demonstrated the cooperative effect of the ER and FOXA1 transcription factors in the estrogen-regulated induction of RET/PTC kinase gene expression via the upstream ERE and FHRE sites. Furthermore, we demonstrated that estrogen upregulates RET/PTC kinase expression and activation of multiple kinase signaling transduction pathways, and that abrogation of RET/PTC kinase expression inhibits estrogen-induced breast cancer cell proliferation. Based on these results, we propose a novel model of regulation and function of RET/PTC kinase in breast cancer (Figure 8). In this model, in breast cancer cells exposed to estrogen, estrogen binds ER and the ER and FOXA1 transcription factors are recruited to the upstream ERE and FHRE sites. These two transcription factors cooperate to increase the transcription of the RET/PTC kinase gene, resulting in upregulated RET/PTC kinase protein expression. In turn, this increased RET/PTC kinase expression augments phosphorylation of downstream kinases, thereby leading to cell proliferation. Finally, abrogation of this signal transduction cascade can inhibit cell proliferation. This model is in accordance with the survival data observed in clinic that high RET/PTC kinase expression is associated with poor prognosis in patients with ER-positive tumors (Figure 1E).

While estrogen and its receptors regulate a variety of genes that are implicated in the development and progression of breast cancer, many estrogen-regulated genes do not contain consensus EREs within their promoters. The molecular mechanisms by which estrogen regulates expression of these genes remains to a great extent unresolved. In previous studies by us and others, several transcription factors (*e.g.* AP-1 [7], Sp1 [8], and NF κ B [9]) have been found to crosstalk with ER and mediate the effects of estrogen regulation of target gene expression. Moreover, FOXA1 has recently been identified as a key determining factor influencing the interaction between ER and chromatin [36, 37]. Thus, the crosstalk between ER and other transcription factors in regulation of critical genes in breast cancer is an important emerging area.

Although RET/PTC kinase is a newly identified estrogen-dependent tyrosine kinase, there are other tyrosine kinases that have been intensively investigated in the field of breast cancer. Particularly well documented is the crosstalk between estrogen and the IGF/EGF

signaling pathways. For example, estrogen induction of type 1 insulin-like growth factor receptor (IGF1R) expression resulted in enhanced tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) after IGF-I stimulation, followed by enhanced MAPK activation [38, 39]. In addition, the crosstalk between ER and the epidermal growth factor receptor (EGFR)/ v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2/HER2/neu) signaling pathways are implicated in tamoxifen resistance [40–42].

Of particular interest is that the crosstalk between estrogen and kinase signaling pathways has potential therapeutic implications. Recent studies have shown that inhibition of both estrogen and the kinase signaling pathways synergistically induces apoptosis in breast cancer animal models [43]. Therefore, coadministration of tyrosine kinase inhibitor and ER inhibitor could have beneficial effects that exceed those observed with either agent independently. Some tyrosine kinase inhibitors (e.g. imatinib [44], sorafenib [45], sunitinib [46]) can efficiently, although nonspecifically, inhibit RET/PTC kinase. Currently, however, inhibitors that specifically target RET/PTC kinase remain unavailable. Therefore, considering the newly identified importance of RET/PTC kinase in breast cancer proliferation, the investigation and discovery of novel highly-specific RET/PTC kinase inhibitors is a high priority.

In summary, the study presented here demonstrates that ER and FOXA1 crosstalk regulates the expression of RET/PTC kinase and that this kinase is a critical regulator of breast cancer cell growth. Additionally, the RET/PTC kinase may also serve as a novel prognostic biomarker for breast cancer patient survival. This study provides novel insights into the regulation and function of this important kinase in breast cancer. Furthermore, the identified “ER-FOXA1-kinase” signaling axis offers potential therapeutic targets for the prevention and treatment of breast cancer in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the NIH grant R01-CA123246. The authors thank Michelle Savage for proofreading and editing the manuscript.

Abbreviations

ER	estrogen receptor
ERE	estrogen-response element
FHRE	forkhead responsive element
RET/PTC kinase gene	rearranged during transfection/papillary thyroid carcinoma
MEN2A	multiple endocrine neoplasia type 2A
ActD	actinomycin D
CHX	cycloheximide
ChIP	chromatin immunoprecipitation
siRNA	small interfering RNA

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Figure 1.

RET/PTC kinase gene expression and its significance in breast cancer. (A) RET/PTC kinase mRNA expression in breast cancer cell lines as determined by qRT-PCR. (B) RET/PTC kinase protein expression in breast cancer cell lines as determined by Western blot. (C) Comparison of RET/PTC kinase mRNA expression in all ER-positive and ER-negative cell lines included in this study. (D) RET/PTC kinase mRNA expression in breast tumor tissue specimens from the three largest independent studies including a total of 625 patients. Data are from the clinical microarray datasets [Bittner *et al*/IGC dataset, Not Published, 2005; Desmedt *et al*/TRANSBIG dataset [25]; Wang *et al*/Veridex dataset [26]]. (E) The correlation between ER and RET/PTC kinase expression in breast tumor tissue specimens

[25]. (F) Kaplan-Meier analysis showing the overall survival of those ER-positive patients whose tumors had either significantly high or significantly low RET/PTC kinase expression [25].

Figure 2.

Effect of estrogen on RET/PTC kinase gene expression. (A) E2 regulation of RET/PTC kinase expression in a panel of breast cancer cell lines. Hormone-depleted cells were treated with vehicle (V) or estrogen (E) for 6h and mRNA expression was analyzed by qRT-PCR. (B) Time course of E2 induction of RET/PTC kinase expression in MCF-7 cells. Inset: Western blot showing E2 induction of RET/PTC kinase expression in MCF-7 cells at 24h of E2 treatment. (C) Effects of Actinomycin D (ActD) and Cycloheximide (CHX) on E2 induction of RET/PTC kinase expression. Hormone depleted MCF-7 cells were treated with ActD (1 μ g/ml) or CHX (10 μ g/ml) for 1h before the addition of vehicle or E2. RNA was harvested 3h after treatment with vehicle or E2. (D) Effect of ICI 182,780 (ICI) on E2

induction of RET/PTC kinase expression. Hormone-depleted MCF-7 cells were treated with ICI (1 μ mol/l) for 1h before the addition of vehicle or E2, and RNA was harvested 3h after treatment with vehicle or E2. (E) Effects of siRNA against ER on RET/PTC kinase expression. Hormone-depleted MCF-7 cells were transfected with siRNA against luciferase (Luc) or estrogen receptor (ER) for 36h before the addition of vehicle or E2. RNA was harvested at 3h after treatment with vehicle or E2. Inset: verification of ER knockdown by Western blot.

Figure 3.

Luciferase assay of RET/PTC kinase gene enhancer/promoter luciferase constructs. (A) Effect of E2 on the reporter activity of various lengths of RET/PTC kinase gene promoter-luciferase constructs. Hormone-depleted MCF-7 cells were transfected with RET/PTC kinase gene promoter luciferase plasmid. Sixteen hours later, cells were treated with vehicle or E2 for 6h and lysed for luciferase analysis. (B) More complete map of RET/PTC kinase gene structure in the genomic context, showing the ER binding sites that were previously identified by eight independent studies (chromosome 10: 42841683 ~ 42842495). (C) Effect of E2 on the reporter activity of RET/PTC kinase promoter luciferase construct with or without the upstream enhancer.

(A)

(B)

Figure 4.

Crosstalk between ER and FOXA1 in regulating RET/PTC kinase gene expression. (A) The predicted potential transcription factor binding sites in the upstream RET/PTC kinase gene enhancer, showing the two estrogen responsive elements (EREs), the single ERE half site (hERE), and the two forkhead responsive elements (FHREs). (B). Effect of E2 on the reporter activity of RET/PTC kinase gene enhancer-promoter luciferase constructs with mutations in the ERE and FHRE sites. (C) Effect of siRNA knockdown of FOXA1 on estrogen-induced RET/PTC kinase gene expression. Hormone-depleted MCF-7 cells were transfected with siRNA against FOXA1. Thirty-six hours later, cells were treated with vehicle or E2 for 3h and harvested for qRT-PCR. Inset: Validation of FOXA1 siRNA on ER

and FOXA1 expression by Western Blot. (D). Effect of FOXA1 overexpression on estrogen-induced RET/PTC kinase gene expression. Hormone-depleted MCF-7 cells were transfected with vector (vec) or CMV-FOXA1 plasmid for 36h and then treated with vehicle or E2 for 3h. RNA was harvested and analyzed by qRT-PCR. Inset: Validation of FOXA1 overexpression by Western blot.

Figure 5. ChIP assay showing recruitment of transcription factor proteins to the RET/PTC kinase gene enhancer. (A). Schematic representation of the ChIP primers used to amplify the RET/PTC kinase enhancer and promoter regions. (B-D). Recruitment of ER, FOXA1, and Pol II proteins, respectively, to the RET/PTC enhancer and promoter regions. Hormone-depleted MCF-7 cells were treated with vehicle or E2 for 30 min and then fixed with formaldehyde. Data were presented as relative amount of immunoprecipitated DNA normalized to input as measured by qPCR assay.

Figure 6.

Effect of estrogen upregulation and siRNA knockdown of RET/PTC kinase expression on downstream kinase signaling pathways. Hormone-depleted MCF-7 cells were transfected with siRNA against RET/PTC kinase or siRNA against luciferase for 36h. Then cells were switched into serum free IMEM with E2 or vehicle for 24h. Before harvesting, cells were treated with physiological level (1ng/ml) of the RET/PTC kinase ligand glial cell line-derived neurotrophic factor (GDNF) for 10min to stimulate the RET/PTC kinase signaling pathway. Western blot analysis of both phosphorylated and unphosphorylated kinase signaling pathway components is shown.

Figure 7.

Effect of estrogen upregulation and siRNA knockdown of RET/PTC kinase expression on breast cancer cell proliferation. (A) Upper Panel: MCF-7 cell proliferation as examined by MTS assay. Lower Panel: verification of RET/PTC kinase knockdown by qRT-PCR. (B) Upper Panel: MDA-MB-453 cell proliferation as examined by MTS assay. Lower Panel: verification of RET/PTC kinase knockdown by qRT-PCR. (C) Upper Panel: MCF-7 cell proliferation as examined by soft agar colony formation assay. Bar: 200μm. Lower Panel: A depiction of the colony counts.

Figure 8. Schematic representation of the ER and FOXA1 transcription factors regulating RET/PTC kinase expression, the associated downstream kinase signaling pathways, and ultimately, breast cancer cell proliferation.