Steroid hormone modulation of RET through two estrogen responsive enhancers in breast cancer

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RET, a gene causatively mutated in Hirschsprung disease and cancer, has recently been implicated in breast cancer estrogen (E2) independence and tamoxifen resistance. RET displays both E2 and retinoic acid (RA)-dependent transcriptional modulation in E2-responsive breast cancers. However, the regulatory elements through which the steroid hormone transcriptional regulation of RET is mediated are poorly defined. Recent genome-wide chromatin immunoprecipitation-based studies have identified 10 putative E2 receptor-alpha (ESR1) and RA receptor alpha-binding sites at the RET locus, of which we demonstrate only two (RET – 49.8 and RET + 32.8) display significant E2 regulatory response when assayed independently in MCF-7 breast cancer cells. We demonstrate that endogenous RET expression and RET – 49.8 regulatory activity are cooperatively regulated by E2 and RA in breast cancer cells. We identify key sequences that are required for RET – 49.8 and RET + 32.8 E2 responsiveness, including motifs known to be bound by ESR1, FOXA1 and TFAP2C. We also report that both RET – 49.8 regulatory activity and endogenous RET expression are completely dependent on ESR1 for their (E2)-induction and that ESR1 is sufficient to mediate the E2-induced enhancer activity of RET – 49.8 and RET + 32.8. Finally, using zebrafish transgenesis, we also demonstrate that RET – 49.8 directs reporter expression in the central nervous system and peripheral nervous system consistent with the endogenous ret expression. Taken collectively, these data suggest that RET transcription in breast cancer cells is modulated by E2 via ESR1 acting on multiple elements collectively.

INTRODUCTION

The RET gene encodes a critical receptor tyrosine kinase required for the genesis and function of neuroendocrine, neuronal and renal populations. As such, its expression is tightly controlled throughout development and postnatal life. During vertebrate embryogenesis, RET is expressed in discrete subsets of neurons in the central and peripheral nervous systems and in the forming kidneys (1–3). RET gain-of-function mutations result in multiple endocrine neoplasia type 2 (4). In contrast, loss of function mutations in RET underlie Hirschsprung disease, a congenital defect marked by enteric aganglionosis (5). RET enhancer mutations have also been implicated in Hirschsprung disease risk (6,7).

Additional evidence suggests a potentially important role for RET in breast cancer (8–10). While, similar to other tyrosine kinases, RET mutations are rarely identified in breast cancer (11), RET over-expression with a corresponding increase in protein levels is detected in a subset of estrogen receptor-alpha (ESR1) positive invasive breast cancers (8,12). Further studies showed increased RET expression positively correlates with ESR1 expression in breast cancer (13,14). Additionally, RET-dependent Glial cell line-derived neurotrophic factor signaling in ESR1-positive breast cancer cell lines causes increased cell scattering and anchorage-independent proliferation (12,13). RET rearrangements were also detected in 8 out of 62 high-grade invasive ductal breast cancers (15), and furthermore expression of the thyroid cancer-associated RET/PTC1 fusion protein led to
mammary gland carcinoma in \( \sim 18\% \) of transgenic mice (16). RET signaling has also been implicated in breast cancer estrogen (E2) independence and tamoxifen resistance, potentially through ESR1 phosphorylation causing ligand-independent transcriptional regulation (17,18). These data strongly suggest a role for RET and its modulation by E2 in breast cancer progression. Further, the ligand independent activation of ESR1 by the ESR1 target gene RET opens the possibility of an autoregulatory loop.

Consistent with the potential role of RET in breast cancer and the correlation of RET expression with ESR1 status, a growing body of data suggests that E2 and retinoic acid (RA) play significant roles in modulating RET transcription during development and disease (19–24). Both E2 and RA modulate transcription through their cognate nuclear receptors [ESR1 and RA receptor-alpha (RARA) (25,26)]. Both E2 and RA have been reported to regulate RET expression in the developing kidney (19,21,27). RA has also been shown to regulate RET expression in neuroblastoma and interact with RET signaling in the enteric nervous system (22,23,28).

Upon RA exposure in the MCF-7 E2 responsive breast cancer cell line, RET is transcriptionally upregulated (29). Similarly, RET is upregulated by E2 exposure in MCF-7 cells (20,30). Although ESR1- and RARA-binding sites often overlap and mediate antagonistic effects on gene regulation in MCF-7 cells, RET is one of a small minority of genes that is upregulated by both RA and E2 (29,30). Furthermore, recent chromatin immunoprecipitation (ChIP)-based studies identified ESR1- and RARA-binding sites genome-wide in MCF-7 cells (20,29–33), including 10 ESR1-binding sites and two RARA-binding sites within and flanking RET. Although the declining cost of high throughput sequencing has allowed for a large increase in ChIP-Seq data, much follow up work remains to aid in the interpretation of whole genome data. Although regulatory element at the RET locus has been studied extensively (34–36), to date no RET E2 response element has been identified. Additionally, no breast cancer RET locus RA response elements (RAREs) have been reported to date.

One recent study described chromatin interactions elicited by E2 stimulation in MCF-7 cells using chromatin interaction analysis by paired end tagging sequencing (ChIA-PET) (37). Their results suggest that E2 signaling is frequently mediated through long-range chromatin interactions, including one interaction unit at the RET locus extending over 89 kb and involving five identified ESR1-binding sequences. Although commonly accepted models of enhancer–promoter interaction involve looping of intervening DNA to place an enhancer in proximity to its cognate promoter (38,39), we know little of the potential consequences of two or more enhancers acting in concert at a common promotor. Some data suggest a simple model of additive autonomy (40), while other data suggest enhancers display cooperativity that is greater than additive (37,41,42).

Despite the growing body of evidence indicating a role for RET in breast cancer, little is known about the sequences that mediate the transcriptional response of RET to steroid hormone super-family members, or how they mediate this effect. We report the characterization of two potent RET locus E2 responsive elements (EREs, \( \text{RET} \rightarrow 49.8 \) and \( \text{RET} \rightarrow 32.8 \)) found by testing the 10 ESR1-binding sites identified by recent whole genome ChIP assays (20,30,31,33). RET \( \rightarrow 49.8 \) also contains a RARA-binding site (29) and similarly demonstrates RA responsiveness. We report that endogenous RET expression and RET \( \rightarrow 49.8 \) directed reporter expression are cooperatively regulated by E2 and RA. We identify several key motifs required for the E2 responsiveness of RET \( \rightarrow 49.8 \) and RET \( \rightarrow 32.8 \). Additionally, the collective regulatory activity of five interacting RET locus ESR1-binding sites can be explained by the activity of RET \( \rightarrow 49.8 \) and RET \( \rightarrow 32.8 \). Finally, we demonstrate the human RET \( \rightarrow 49.8 \) sequence directs RET appropriate neuronal regulatory control when assayed in transgenic zebrafish embryos.

**RESULTS**

**Identification of E2 and RA responsive elements at RET**

We set out to determine the E2 and RA responsiveness of sequences encompassing ChIP-identified ESR1- and RARA-binding sites at the RET locus. We defined the corresponding locus as the \( \sim 300 \) kb interval encompassing RET and flanked by BMS1 and CSGALNACT2 (chr10:42653245-42948563; hg18). Taken collectively, recent genome-wide MCF-7 ChIP-based studies have identified 10 RET locus ESR1-binding sites \( \{ \text{RET} \rightarrow 94.4, \text{RET} \rightarrow 49.8, \text{RET} \rightarrow 38.1, \text{RET} \rightarrow 31.7, \text{RET} \rightarrow 15.8, \text{RET} \rightarrow 8.7, \text{RET} \rightarrow 4.8, \text{RET} \rightarrow 9.7, \text{RET} \rightarrow 28.1 \text{ and} \text{RET} \rightarrow 32.8 \}; \) Figure 1A; Supplementary Material, Tables S1 and S2; (20,30–33), all named here according to their distance from the RET transcriptional start site, and two RET locus RARA-binding sites (RET \( \rightarrow 49.8 \) and RET \( \rightarrow 23 \)). We cloned sequences encompassing these sites individually into a luciferase reporter vector and assayed their capacity to modulate reporter levels in response to E2 (17-estradiol) and RA (all-trans RA) in transfected MCF-7 cells (Fig. 1B and C). Of the 10 identified ESR1-binding sites, only RET \( \rightarrow 49.8 \) and RET \( \rightarrow 32.8 \) displayed significant E2 responsiveness, exhibiting \( \sim 10 \)- and 7-fold upregulation, respectively, versus the promoter-only construct in response to E2 treatment (Fig. 1B; \( \text{RET} \rightarrow 49.8, P\text{-value} <0.001; \text{RET} \rightarrow 32.8, P\text{-value} <0.001 \)). Consistent with these data, RET \( \rightarrow 49.8 \) and RET \( \rightarrow 32.8 \) also show E2 responsiveness when assayed in another ESR1-positive breast cancer cell line (T47D, Supplementary Material, Fig. S1A). Similarly, of the two identified RARA-binding sites, only RET \( \rightarrow 49.8 \) displayed upregulation in response to RA in MCF-7 cells (Fig. 1C, 2.7-fold, \( P \) value <0.005). These data demonstrate that while RET \( \rightarrow 32.8 \) acts only as an ERE in these assays, RET \( \rightarrow 49.8 \) can behave as both an ERE and a RARE.

**RET \( \rightarrow 49.8 \) exhibits greater than additive response to combined E2 and RA treatment**

Given the recent reports of crosstalk between the E2 and RA signaling pathways (29) and our observation that the RET \( \rightarrow 49.8 \) sequence responds positively upon exposure to both factors independently, we similarly assayed RET \( \rightarrow 49.8 \) luciferase reporter response to combined E2 and RA treatment in MCF-7 cells. When treated with E2 and RA in combination, RET \( \rightarrow 49.8 \) upregulated reporter expression synergistically
Endogenous RET is cooperatively regulated by E2 and RA signaling

Given the synergistic activation of RET −49.8 by E2 and RA signaling, we then assayed the effects of the combined treatment of E2 and RA on endogenous RET gene expression in MCF-7 cells (Fig. 2B). Consistent with previous reports (20,29,43), RET was upregulated in MCF-7 cells following 24 h of E2 treatment (Fig. 2B; 7.4-fold RET upregulation versus hormone deprived condition, P-value <0.0001) or 24 h of treatment with RA (Fig. 3A; 1.6-fold versus hormone deprived condition, P-value <0.001). Simultaneous treatment with E2 and RA upregulated of RET in a manner consistent with a cooperative model (multiplicative) (Fig. 2B; 18-fold in RA + E2 versus 7.4-fold in E2 treated and 1.6-fold in RA treated), but not synergistic upregulation (test for whether combined effect is greater than multiplicative has P-value >0.15). Although the magnitude of the combined response differs, both RET and the RET −49.8 construct display greater than additive responses to RA and E2 in combination. The difference observed in their magnitude of response may simply reflect the evaluation of RET −49.8 out of genomic context using a heterologous promoter.

Identification of key motifs for RET −49.8 E2 responsiveness

We then set out to identify sequences within RET −49.8 required for its E2 responsive regulatory activity. Intervals within the ERE RET −49.8 have been reported to bind ESR1 as well as the pioneer factor FOXA1 (30,37,44). Additionally, RET −49.8 can be bound by TFAP2C, which regulates RET and many other parts of the E2 receptor signaling pathway. Using published transcription factor position weight matrices, we identified two FOXA1-like and two TFAP2C motifs overlapping previously published MCF-7 DNase hypersensitivity sites, a robust predictor of transcription factor binding [(45–48); UW ENCODE Group; Fig. 3A]. Furthermore, we also selected a 9 bp putative critical region (CR) and a 12 bp putative CR within RET −49.8.
identified previously as ESR1-binding sites by ChIP-Seq-based studies [9 bp CR (30) and 12 bp CR (37)].

We then mutated the identified motifs to determine their effect on \( \text{RET} - 49.8 \) regulatory activity. All \( \text{RET} - 49.8 \) constructs (wild-type and mutants) were assayed for their effects on E2 response (Fig. 3B). Mutation of either FOXA1-like motif (FOXA1 S1 and S2) resulted in the near loss of \( \text{RET} - 49.8 \) reporter responsiveness to E2 (Fig. 3B; FOXA1 S1 and S2 \( P \)-value for reduction versus wild-type \( < 0.004 \)). Further, mutation of the 9 bp CR and the 12 bp CR both resulted in significantly reduced but not abrogated E2 response (Fig. 3B; 12 bp CR \( P \)-value for reduction versus wild-type \( < 0.05; 9 \) bp CR \( P \)-value \( < 0.01 \)), suggesting there may be redundancy in ESR1-binding sites. Mutagenesis of either TFAP2C site (TFAP2C S1 and TFAP2C S2) in the \( \text{RET} - 49.8 \) luciferase vector led to highly reduced E2 response (Fig. 3B; TFAP2C S1 and TFAP2C S2 \( P \)-value for reduction versus wild-type \( < 0.001 \)). These data suggest that the 9 bp CR, 12 bp CR, TFAP2C-like motifs and FOXA1-like motifs are critical components of \( \text{RET} - 49.8 \) regulatory control in response to E2 (32).

**Identification of key motifs for \( \text{RET} + 32.8 \) E2 responsiveness**

Having identified sequences critical for independent \( \text{RET} - 49.8 \) function, we similarly set out to test the necessity of sequences within \( \text{RET} + 32.8 \) E2 responsiveness. \( \text{RET} + 32.8 \) is bound by TFAP2C and ESR1, but not FOXA1 or RARA (26,29,37,44,49). We identified an ESR1 motif in the ESR1-binding peak and a TFAP2C motif in the TFAP2C-binding peak within an MCF-7 DNase hypersensitivity site (TFAP2C S3) by integrating published motifs and the rVISTA2.0 tool [http://rvista.dcode.org/; Fig. 3C; UW Encode Group; (47,49,50)]. While the wild-type \( \text{RET} + 32.8 \) showed E2 responsiveness, mutagenesis of the ESR1 motif or the TFAP2C S3 motif led to significant reduction in E2 response (Fig. 3D; ESR1 mutant \( P \)-value versus wild-type \( < 0.001 \), TFAP2C S3 mutant \( P \)-value versus wild-type \( < 0.001 \)), indicating a critical role for those motifs in \( \text{RET} + 32.8 \) function.

**ESR1 is necessary for E2-mediated transcriptional modulation of \( \text{RET} \) and \( \text{RET} 49.8 \) response elements**

Since \( \text{RET} + 49.8 \) is a primary target of E2 signaling and ESR1 binds \( \text{RET} - 49.8 \) and \( \text{RET} + 32.8 \), we tested whether ESR1 is necessary for E2-mediated modulation of endogenous \( \text{RET} + 49.8 \) expression and \( \text{RET} - 49.8 \) and \( \text{RET} + 32.8 \) regulatory activity in MCF-7 cells using siRNA-mediated knockdown (Fig. 4A and F). Unlike treatment with control non-targeting siRNA, ESR1 siRNA treatment resulted in a complete abrogation of E2-induced \( \text{RET} + 49.8 \) upregulation (Fig. 4A; \( P \)-value \( \sim 0.52 \)). Similarly, treatment with ESR1 siRNA almost completely prevents \( \text{RET} - 49.8 \) and \( \text{RET} + 32.8 \) directed reporter E2 responsiveness (Fig. 4B). Treatment with the selective E2 receptor modulator tamoxifen also reduced E2 responsiveness of \( \text{RET} - 49.8 \) (Supplementary Material, Fig. S2), taken together with the effect of ESR1 siRNA suggests that ESR1 is critical and dependent on its ligand for eliciting the \( \text{RET} - 49.8 \) E2 response. Additionally, consistent with its role as an ESR1 pioneer co-factor (44) that binds \( \text{RET} - 49.8 \), FOXA1 siRNA knockdown reduced but did not completely eliminate endogenous \( \text{RET} 49.8 \) E2 responsiveness (Fig. 4C, 1.6-fold versus E2-deprived non-targeting siRNA). Similar to endogenous \( \text{RET} + 49.8 \) expression, FOXA1 knockdown reduced but did not abrogate \( \text{RET} - 49.8 \) E2 responsiveness (Fig. 4D). These data support the model that \( \text{RET} - 49.8 \) and \( \text{RET} + 32.8 \) act as ESR1-dependent EREs in a manner consistent with endogenous \( \text{RET} + 49.8 \) expression and raises the question of whether ESR1 is sufficient for this activity.
ESR1 is sufficient to induce RET-49.8 and RET+32.8 directed E2 responsiveness in ESR1-negative MDA-MB-231 cells

We tested the sufficiency of ESR1 to facilitate E2-induced RET-49.8 and RET+32.8 regulatory activity using the MDA-MB-231 (human ESR1-negative breast cancer) cell line (Fig. 4E and H) (51). E2 treatment did not induce increased luciferase activity in cells co-transfected with RET-49.8 or RET+32.8 luciferase vector and an empty expression vector (pCDNA3.1 empty) (Fig. 4E; RET-49.8 pCDNA 3.1 empty P-value >0.10; RET+32.8 pCDNA 3.1 empty P-value >0.25). In contrast, E2 treatment of cells co-transfected with the RET-49.8 luciferase vector and a plasmid constitutively expressing ESR1 (pCDNA3.1 ESR1; Fig. 4H) induced luciferase activity at levels ~20-fold higher than the promoter-only construct (Fig. 4E; P-value versus promoter <0.001). Similarly, E2 treatment of cells co-transfected with RET+32.8 and pCDNA3.1 ESR1-induced luciferase activity at levels ~14-fold higher than the promoter-only construct (P-value versus promoter <0.002).

Taken collectively, these data further support a model in which ESR1 is necessary and sufficient for E2-induced enhancer activity of RET-49.8 and RET+32.8.

Enhancer activity of five interacting RET locus
ESR1-binding sites does not exceed RET-49.8 and RET+32.8 in combination

Genome-wide chromatin interaction analyses in MCF-7 cells (52) identified E2-induced interactions between five ESR1-binding sites at the RET locus (RET-49.8, RET-38.1, RET-31.7, RET+4.8 and RET+32.8). Of these elements, we have already shown that only RET-49.8 and RET+32.8 display significant E2-induced regulatory activity when assayed independently (Fig. 1B). We then set out to determine whether physical co-localization of these elements corresponded to collective regulatory activity that exceeds their independent output, constructing a luciferase vector containing all five interacting elements, or subsets of the elements (Fig. 5). While RET-49.8 and RET+32.8 showed independent regulatory activity as before (~12-fold versus promoter and ~7-fold versus promoter E2 response, respectively), a
**RET**−49.8 and RET−32.8 are dependent on ESR1. (A) Quantitative real-time PCR assay of the effects of ESR1 knockdown using siRNA on endogenous MCF-7 RET expression, normalized to 18S rRNA. RET expression levels for E2-deprived conditions were normalized as 1-fold expression. NT siRNA, non-targeting control siRNA; E2-deprived, gray bars; E2-treated, black bars. (B) Effects of ESR1 knockdown via siRNA on pDSMA RET−49.8 and RET−32.8 E2 responsiveness in MCF-7 cells (NT siRNA, non-targeting control siRNA). (C) Quantitative real-time PCR assay of the effects of FOXA1 knockdown using siRNA on endogenous MCF-7 RET expression, normalized to 18S rRNA. RET expression levels for E2-deprived conditions were normalized as 1-fold expression. NT siRNA, non-targeting control siRNA; E2-deprived, gray bars; E2-treated, black bars. (D) Effects of FOXA1 knockdown via siRNA on pDSMA RET−49.8 E2 responsiveness in MCF-7 cells (NT siRNA, non-targeting control siRNA). (E) Effects of FOXA1 knockdown using siRNA on pDSMA RET−49.8 E2 responsiveness in MCF-7 cells (NT siRNA, non-targeting control siRNA). (F) Western blot confirms ESR1 knockdown in MCF-7 cells co-transfected with targeting or non-targeting (NT) siRNA co-transfected with pDSMA RET−49.8. (G) Western blot confirms FOXA1 knockdown in MCF-7 cells co-transfected with targeting or non-targeting (NT) siRNA co-transfected with pDSMA RET−49.8. (H) Western blot images demonstrating expression of ESR1 only in MDA-MB-231 cells transfected with pCDNA3.1 empty and pCDNA3.1 ESR1.

**RET**−49.8 exhibits RET appropriate in vivo regulatory activity

To determine whether the identified EREs (RET−49.8 and RET−32.8) also displayed in vivo regulatory control in addition to their role in E2-responsiveness, we assayed their regulatory activity using Tol2-based stable transgenesis in zebrafish (35,52). While RET−32.8 did not exhibit in vivo regulatory activity at the times assayed (1–4 days post-fertilization), RET−49.8 directed broad central nervous system expression of enhanced green fluorescent protein (eGFP) (Fig. 6A), overlapping the expression domains of endogenous ret in the central nervous system at 24 hpf (1). By 72 hpf, RET−49.8 directed discrete reporter expression in the dorsal spinal cord, consistent with the position of the ret expressing Rohon-Beard neurons and interneurons (dorsal; Fig. 6B) (1,53); a dense column of ventral spinal cord consistent with motor neurons (53) and in the ret-positive lateral line ganglia (1). Consistent with its activity in neuronal populations and with the endogenous expression of ret, RET−49.8 acted as a classical enhancer in SK-N-SH human neural crest-derived neuroblastoma cells, directing luciferase expression almost 2-fold greater than the promoter-only construct (Supplementary Material, Fig. S3A). Additionally, RET−49.8 maintains its ability respond to E2, in both SK-N-SH cells and mouse neuroblastoma Neuro2A cells, when co-transfected with an ESR1 expression vector (Supplementary Material, Fig. S3A and B). Taken collectively, these data suggest that RET−49.8 may act as a classical enhancer that may be modulated by E2 in a tissue and stage-dependent manner.
Figure 5. RET locus interacting ESR1-binding sites do not exceed the activity of RET −49.8 and RET +32.8. The ESR1-binding sites present in each vector are represented graphically on the left of each numbered construct. Light gray box represents SV40 promoter, white box represent luciferase coding region, while black boxes represent RET locus ESR1-binding sites from left to right RET −49.8, RET −38.1, RET −31.7, RET +4.8 and RET +32.8. E2 responsive luciferase activity of the different combinations RET locus ESR1-binding sites predicted to interact in MCF-7 cells (37). E2-depleted (gray bars) and E2-exposed (10 nM; black bars) pDSMA RET expression values were normalized against a promoter-only construct (Prom. only), pDSMA construct. Constructs are labeled 1 through 10. (1) Promoter-only construct; (2) RET −49.8; (3) RET −38.1; (4) RET −31.7; (5) RET +4.8; (6) RET +32.8; (7) RET −49.38-31+4;32; (8) RET −49.38-31; (9) RET −49.44+32; (10) RET −49.32; all assays were conducted in triplicate (error bars, SD).

DISCUSSION

RET is a developmentally critical gene. Its transcription has been well documented in many cell populations and is modulated by E2 and RA during development and disease (13,14,20–22,24,29,30,43). We believe this is the first study to characterize specific E2 response elements at this critical locus, as well as the first breast cancer RARE. Here we have studied 10 ESR1-binding sequences and 2 RARA-binding sequences at RET, identifying one particularly potent ERE and RARE (RET −49.8) and characterizing it in detail. Additionally, we identified and characterized a second RET locus E2 response element (RET +32.8).

We demonstrate the dependence of the endogenous RET expression and both RET −49.8 and RET +32.8 regulatory activity on ESR1 for E2 modulation. We further identify multiple sequence motifs within RET −49.8 that are required for its E2-induced activity. These sites include two FOXA1-like motifs and 12 bp and 9 bp CR sequences identified by ChIP-PET and ChIP-SEQ as ESR1 CRs (12 bp CR and 9 bp CR) (30,37). We have also shown the dependence of RET −49.8 and RET +32.8 on TFAP2C-like motifs for E2 response. Furthermore, RET −49.8 binds both cohesin and RNA polymerase II in MCF-7 cells (54,55) consistent with other endogenous enhancers (51,52) and supporting the idea that these sequences play regulatory roles in their genomic context.

Although E2 and RA signaling display antagonistic effects on most genes they co-modulate in MCF-7 cells (29), we observe that RET transcription appears to be cooperatively controlled by these factors in these cells. Consistent with this observation, RET −49.8 is synergistically upregulated by both E2 and RA (Fig. 2), suggesting a potentially pivotal role for RET −49.8 in integrating E2 and RA signaling. Additionally, sequence within RET −49.8 has been shown in MCF-7 cells to bind E2F1, a cell cycle regulator, and TFAP2C [Yale/UC Davis/Harvard ENCODE Group; (47,49)], both of which have been implicated in hormone therapy resistance in breast cancer (56–59). RET −49.8 has also been shown to bind the ESR1 co-factor SRC-3 (60,61). Additionally, the E2 responsiveness of RET −49.8 and RET +32.8 in T47D E2 responsive breast cancer cell lines suggests that these elements could be functional in multiple E2 receptor-positive breast cancers (55). Given the established role for these pathways in breast cancer, these data may also indicate a role for RET −49.8 and RET +32.8 and the factors they interact with in breast tumor progression.

After characterizing their regulatory responses in vitro, we then establish that RET −49.8, but not RET +32.8, has the independent capacity to act as an in vivo classical tissue-dependent enhancer consistent with the expression of the zebrafish RET ortholog (retl) (1). Whether this in vivo activity is also dependent on ESR1, RARA or another factor remains to be determined. Here we observe three classes of ESR1-binding sites; an E2-modulated tissue-specific in vivo enhancer, an in vitro ERE with no overt independent in vivo function in our transgenic assay and a three sequences that bind ESR1.
and are brought into physical proximity in response to E2 but have no overt function in either our in vitro or in vivo assays. The observation that \( RET-49.8 \) functions as both a \( RET \) ERE in MCF-7 cells and as a neuronal enhancer in vitro and in vivo suggests a potentially broader role for ERE and RARE potentially modulating classical enhancer activity. Furthermore, the final class of sequences may be postulated to recruit transcription factors or stabilize loop formation making a contribution to transcriptional control that is not overt when assayed independently. Either way, these data suggest that our expectations of what comprise biologically valuable sequences may need to be reevaluated as the range of assays to which they are subjected become progressively deeper. In the context of E2-RA-based transcriptional control, although thousands of ESR1 and RAR-binding sites have been identified in breast cancer cell lines, their in vitro and in vivo regulatory activities remain largely untested.

Our data suggest that \( RET-49.8 \) and \( RET+32.8 \) are likely to be required for E2-mediated \( RET \) transcriptional response in breast cancer. Additionally, the crosstalk between RA and E2 signaling has potential therapeutic implications for the treatment of breast cancer (29). Consequently, regulatory elements, such as \( RET-49.8 \), which integrate RA and E2 signaling may hold further insights into breast cancer risk, progression and treatment. Similarly, understanding the disease risk and prognostic consequences of variation within these regulatory elements and the gene products that lie upstream may further refine individual patient management. We are building on this platform to determine the necessity of \( RET-49.8 \) and \( RET+32.8 \) for endogenous E2-mediated \( RET \) transcriptional modulation during development and tumor progression.

MATERIALS AND METHODS

Luciferase reporter assays

Sequences encompassing 10 ESR1-binding sites and 2 RARA-binding sites identified at the \( RET \) locus by whole genome ChIP assay on MCF-7 cells (20,30–33) were amplified and cloned (Primers and intervals; Supplementary Material, Table S2) into pDSMA, a Gateway-modified pGL3 (Promega) (34). The sequences identified in each study are listed in Supplementary Material, Table S1. The constructs were termed pDSMA*RET*, named based on their distance from the \( RET \) transcriptional start site. \( RET-8.7, RET+5.1 \) and \( RET+9.7 \) were previously termed \( RET \) HCS-8.7, \( RET+5.1 \) and \( RET+9.7 \) (34,35). For E2 deprivation MCF-7, T47D, MDA-MB-231, SK-N-SH or Neuro2A cells were grown for 2 days in phenol red free media supplemented with 10% charcoal-stripped fetal bovine serum, then transfected with luciferase vectors containing the binding regions or promoter-only and a renilla control using Lipofectamine 2000 (MCF-7, T47D, SK-N-SH, Neuro2A) or Lipofectamine LTX with Plus reagent (MDA-MB-231) using manufacturer’s protocol. The transfected cells were treated with 10 nm 17β-estradiol (Sigma), 1 μM all-trans RA (Sigma) or 1 μM all-trans RA and 10 nm 17β-estradiol, or E2-deprived media with matching ethanol and then assayed using Dual-Glo Luciferase Kit (Promega).

MCF-7 siRNA luciferase experiment

E2-deprived MCF-7 cells were co-transfected with appropriate pDSMA firefly luciferase plasmid and 6 pM of appropriate Dharmacon SMARTpool siRNA for ESR1, FOXA1 or non-targeting siRNA using Lipofectamine 2000 (Invitrogen). Twenty-four hours after lipofection, the media were removed and cells were treated with ethanol or E2 as described above. Twenty-four hours after the addition of treatment, luciferase activity was performed as described above.

MDA-MB-231 transactivation

Human full-length cDNA clones from the Mammalian Gene Collection (62) for FOXA1 and ESR1 were purchased (Fisher), subcloned into pCND3.1 using Infusion cloning (Stratagene, Primers, Supplementary Material, Table S3). pDSMA \( RET-49.8 \) or pDSMA promoter and a renilla control vector were co-transfected into MDA-MB-231 cells with pcDNA3.1 ESR1 or empty pcDNA3.1 using Lipofectamine LTX with Plus Reagent (Invitrogen). The co-transfected cells were then treated with either E2 or ethanol for 24 h. Luciferase activity was then performed using Promega Dual Glo Luciferase as described above.

Generation of mutagenized pDSMA constructs

Site-directed mutagenesis to convert motifs to EcoRV digest site was performed on motifs of interest using QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene). Primers (listed in Supplementary Material, Table S4) were designed using the QuikChange® Primer Design Program. Serial mutagenesis was used to create double mutants.

MCF-7 quantitative PCR

The E2-depleted cells were treated for 24 h with agonists as described in Luciferase Reporter assays and total RNA collected. cDNA was then synthesized off of total RNA, cDNA synthesized from total mRNA using random hexamers (Super Script III First Strand synthesis; Invitrogen). Quantitative polymerase chain reaction (PCR) was done using Taqman (ABI) probes for \( RET \) and 18S rRNA with Taqman Gene Expression Master Mix (ABI).

siRNA knockdown followed by quantitative PCR

Reverse transfections of 30 picomoles of non-targeting siRNA (Dharmacon) or target gene-directed SMARTpool siRNA into E2 (Dharmacon) deprived MCF-7 were performed using Lipofectamine RNAmax (Invitrogen). Two days after siRNA delivery, agonist treatment was performed for 24 h as described above. Quantitative PCR was then performed as described above on total RNA extracted from siRNA treated MCF-7 cells.

Zebrafish embryo injections and analysis

Sequences studied in luciferase that have not been previously injected into AB zebrafish (Danio rerio) (35) were Gateway
were cloned upstream of the SV40 promoter, while vector construction, all elements located 5′ acting sequences were cloned serially into the pDSMA promoter (35,52,63,64). Reporter expression directed by the described (35,52,63,64). Reporter expression directed by the ESR1-binding amplicons [(37); data set IHM001F]. Pair-wise interactions previously published ESR1-dependent MCF-7 chromatin interlocus interacting sequences were identified from the pre-RET

Design and generation of constructs containing multiple ESR1-binding amplicons

RET locus interacting sequences were identified from the previously published ESR1-dependent MCF-7 chromatin interaction map [(37); data set IHM001F]. Pair-wise interactions were identified between regions containing ESR1-binding sites RET −49.8 and RET −38.1, RET −49.8 and RET −31.7, RET −49.8 and RET +4.8, and RET −49.8 and RET +32.8. Vectors containing different combinations of five interacting sequences were cloned serially into the pDSMA Gateway-modified pGL3 luciferase vector using Infusion cloning (Primers; Supplementary Material, Table S5). During vector construction, all elements located 5′ of the endogenous RET promoter (RET −49.8, RET −38.1, and RET −31.7) were cloned upstream of the of the SV40 promoter, while elements 3′ of the endogenous RET promoter (RET +4.8 and RET +32.8) were cloned 3′ of the Luciferase coding sequence.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflicts of Interest statement. None declared.

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