HER4 selectively coregulates estrogen stimulated genes associated with breast tumor cell proliferation

Wen Han, Frank E. Jones *

Department of Cell and Molecular Biology, Tulane University, New Orleans, LA 70118, USA

ABSTRACT

The EGFR-family member HER4 undergoes regulated intramembrane proteolysis (RIP) to generate an intracellular domain (4ICD) that functions as a transcriptional coactivator. Accordingly, 4ICD coactivates the estrogen receptor (ER) and associates with ER at target gene promoters in breast tumor cells. However, the extent of 4ICD coactivation of ER and the functional significance of the 4ICD/ER transcriptional complex is unclear. To identify 4ICD coactivated genes we performed a microarray gene expression analysis of β-estradiol treated cells comparing control MCF-7 breast cancer cells to MCF-7 cells where HER4 expression was stably suppressed using a shRNA. In the MCF-7 cell line, β-estradiol significantly stimulated or repressed by 2-fold or more 726 or 53 genes, respectively. Significantly, HER4/4ICD was an obligate coactivator for 277 or 38% of the β-estradiol stimulated genes. Ingenuity Pathway Analysis of β-estradiol regulated genes identified significant associations with multiple cellular functions regulating cellular growth and proliferation, cell cycle progression, cancer metastasis, decreased hypoplasia, tumor cell migration, apoptotic resistance of tumor cells, and increased transcription. Genes coactivated by 4ICD displayed functional specificity by only significantly contributing to cellular growth and proliferation, cell cycle progression, and decreased hypoplasia. In direct concordance with these *in situ* results we show that HER4 knockdown in MCF-7 cells results in a loss of estrogen stimulated tumor cell proliferation and cell cycle progression, whereas, estrogen stimulated tumor cell migration was unaffected by loss of HER4 expression. In summary, we demonstrate for the first time that a cell surface receptor functions as an obligate ER coactivator with functional specificity associated with breast tumor cell proliferation and cell cycle progression. Nearly 90% of ER positive tumors coexpress HER4, therefore we predict that the majority of breast cancer patients would benefit from a strategy to therapeutic disengage ER/4ICD coregulated tumor cell proliferation.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

The estrogen receptor (ER) is a nuclear receptor overexpressed in over 70% of primary breast cancers [1]. The biological activity of ER is regulated, in part, through the association with a functionally diverse complex of transcriptional coregulators at target gene promoters. For example, independently suppressed expression of p160 steroid receptor coactivator (SRC) family members results in impaired estrogen stimulated tumor cell proliferation [2,3]. The important contribution of ER associated coactivator complexes to breast tumor cell proliferation is further underscored by the suc-

cess of tamoxifen therapy for the treatment of ER positive breast cancer. Binding of tamoxifen alters ER structure thereby disrupting ER association with coactivators and arresting tumor cell cycle progression [4,5]. Therefore targeting ER coactivators has been suggested as a potential therapeutic strategy for breast cancer [6].

The epidermal growth factor receptor (EGFR) family member, HER4, is a putative ER coactivator with unique properties. HER4 undergoes proteolytic processing at the cell surface to release an independently signaling HER4 intracellular domain (4ICD) with transcriptional coactivator activity [7,8]. For example, 4ICD interacts with ER in response to estrogen and coactivates specific genes by associating with their promoters in a complex with ER [9,10]. Suppression of HER4 expression abrogates estrogen stimulated breast tumor cell proliferation [10–12] raising the possibility that 4ICD coactivation of ER regulates genes associated with tumorigenesis. Accordingly, 4ICD coactivates CXCL12 (SDF-1) [10], an estrogen regulated gene involved in tumor growth and metastasis [13]. Clinically, HER4 expression is significantly associated with ER expression [14] and up to 90% of ER positive

tumors coexpress HER4 [15]. These clinical and experimental observations suggest a strong tumor cell selection for establishing a HER4/ER proliferative signal in breast cancer.

Although substantial evidence supports a role for ER coactivators in breast tumorigenesis, the estrogen regulated genes which serve as coactivator targets contributing to tumor cell proliferation remain to be determined. Here we use microarray gene expression analysis to demonstrate that 4ICD is required for the coactivation of 38% of estrogen regulated genes. Using a systems biology approach and biological assays we further show primary functions for 4ICD coactivated genes in cell cycle progression and tumor cell proliferation. Our results represent the first global gene expression functional analysis for an ER coactivator and establish 4ICD as an important ER coactivator required for estrogen stimulated breast tumor cell proliferation.

2. Materials and methods

2.1. Cell lines

MCF-7 cells were purchased from American Type Culture Collection and cultured according to their instructions. For stable suppression of HER4 to generate the pooled MCF-7/H4Si4 cell line, MCF-7 cells were transfected with the MISSION shRNA plasmid-DNA TRCN0000039690 targeting HER4 (Sigma) using Fugene6 (Roche). The pooled MCF-7/pLKO vector control cell line was generated by transfecting MCF-7 cells with the pLKO.1 plasmid (Sigma) using Fugene6.

2.2. Western blot analysis

Total cell lysates were prepared in RIPA Buffer (10 mM $NaPO_4$, pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Na-deoxycholate, 1% Nonedit P40) containing Complete EDTA-free Protease Inhibitor Cocktail (Roche) and PhosSTOP phosphatase inhibitor (Roche). Lysates were solubilized in NuPAGE LDS Sample Buffer (4X) (Life Technologies) with NuPAGE Sample Reducing Agent (10X) (Life Technologies) added to 1X. Lysates were separated by electrophoresis with 20 μg of protein per lane in a NuPAGE 4–12% Bis–Tris Gel (Life Technologies) and transferred to an Immobilon-FL 0.45 μm Pore Size Transfer Membrane (Millipore) using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Membranes were blocked and all antibody dilutions were performed in 5% bovine serum albumin (BSA) (Sigma) in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20). All washes were performed in TBST. Primary antibodies included ErbB4 (E200) (Abcam) and α-tubulin #05829 (Upstate Biotechnology).

2.3. Microarray gene expression profiling

MCF-7/pLKO and MCF-7/H4Si4 cells were incubated in phenol red-free MEM supplemented with 5% CS-FBS for 48 h then left untreated or treated with 100 pM 17-β-estradiol (Sigma) for 6 h. Total RNA from three independent experiments was purified using the miRVANA RNA Isolation System (Life Technologies) according to the manufacturer's instructions and RNA integrity was confirmed using a Bioanalyzer (Agilent Technologies). Two-color microarray gene expression analysis was performed using the Agilent Technologies DNA Microarray Scanner and associated protocols. Briefly, 100 ng of total RNA was diluted to generate the spike mix using the RNA Spike-In Kit (Agilent Technologies) exactly as described by the manufacturer. Amplified and cyanine 3 (Cy3)- or cyanine 5 (Cy5)-labeled complementary RNA (cRNA) was prepared for each total RNA sample from 2 μl of spike mix and 100 ng of total RNA using the Low Input Quick Amp Labeling Kit (Agilent Technologies)

exactly as described by the manufacturer. The cRNA was purified using the RNeasy Mini Kit (Qiagen) and purified cRNA and Cy3 or Cy5 were quantitated using a NanoDrop 2000 (Thermo Scientific). Using the Gene Expression Hybridization Kit (Agilent Technologies) 825 ng each of Cy3- or Cy5-labeled, amplified, and purified cRNA were hybridized to a single microarray of a Whole Human Genome 4 × 44 K Microarray G4112F (Agilent Technologies) in a SureHyb chamber (Agilent Technologies) and Agilent G2545A Hybridization Oven (Agilent Technologies) at 65 °C for 17 h as described by the manufacturer. Washed microarray slides were scanned in a DNA MicroArray Scanner G2505C (Agilent Technologies). Microarray data was extracted and quality controlled using Feature Extraction software (Version 10.7.1.1) (Agilent Technologies). Statistical analysis of gene expression data comparing triplicate untreated to 17-β-estradiol treated samples was performed using GeneSpring GX (Agilent Technologies). Genes in each dataset with a 2-fold or greater change in expression following 17-β-estradiol treatment ($p < 0.05$) were subjected to Cellular Function Analysis using Ingenuity Pathway Analysis (IPA) software (Version 17199142).

2.4. Quantitative RT-PCR

Cells were preincubated in phenol red-free MEM supplemented with 5% charcoal-stripped FBS (CS-FBS) for 48 h and were left untreated or treated with 100 pM 17-β-estradiol for 6 h. Triplicate total RNA samples were purified using the miRVANA RNA Isolation System according to the manufacturer's instructions and RNA integrity was confirmed using a Bioanalyzer. First-strand complementary DNA (cDNA) was synthesized from 1.0 μg of total RNA in a 20 μl reaction volume using the Superscript III First-Strand Synthesis System (Life Technologies) with random hexamers exactly as described by the manufacturer. Following reverse transcription, 180 μl of DEPC Treated Water (Invitrogen) was added to the cDNA reaction and 2 μl of the diluted cDNA was used in a 20 μl Power SYBR Green PCR Master Mix (Applied Biosystems) with 250 nM of each oligonucleotide primer to amplify GAPDH, TFF1, CXCL12, or PgR described elsewhere [10] or the RASGPR1 oligonucleotide primers 5′-ACATTTAGCCAAAGGAGCCA and 5′-TACT-TCGACACAGGTTTCCA. Reactions were amplified in the 7500 Fast Real-Time PCR system (conditions as follows: 55 °C for 20 min, 95 °C for 10 min and then 40 cycles of 95 °C for 15 s and 60 °C for 60 s), as described by the manufacturer (Applied Biosystems). The CT analysis for each reaction was performed using the supplied 7500 Software v2.0.5 (Applied Biosystems). Gene expression levels were normalized to GAPDH and 17-β-estradiol stimulated expression relative to untreated control was calculated using the $2^{-\Delta\Delta CT}$ method. Each sample was prepared in triplicate and the data represent the mean and standard error (SE) of at least three independent experiments. Statistically significant differences between data sets were determined using paired Student's t test.

2.5. Colony formation assay

Cells were plated at 1000 cells per well in a 6-well plate with phenol red-free MEM supplemented with 5% CS-FBS with or without 10 nM 17-β-estradiol. Media was replaced every 2 days for 12 days total. Colonies were fixed in 100% methanol and stained with crystal violet. Colony number was calculated using a ColCount Colony Counter (Oxford Optronix) and the supplied statistical software. Each sample was prepared in duplicate and the data represent the mean and SE of at least three independent experiments. Statistically significant differences between data sets were determined using paired Student's t test.

2.6. xCELLigence cell proliferation assay

Cell proliferation was determined using the xCELLigence System (Roche) by plating 2000 cells in an E-Plate 16 in the RTCA DP Instrument (Roche) according to the manufacturer's instructions. After 24 h media was changed to phenol red-free MEM supplemented with 5% CS-FBS and after an additional 48 h cells were left untreated or treated with 10 nM 17-β-estradiol. Cell proliferation as a function of real-time changes in electrical impedance, also referred to as cell index, was monitored by the xCELLigence System for 72 h. The slope of the change in cell index over time and the standard deviation of replicates were calculated using the supplied RTCA Software (Roche). Each sample was prepared in triplicate and the data represent the mean and SE of at least three independent experiments. Statistically significant differences between data sets were determined using paired Student's *t* test.

2.7. Cell cycle analysis

Cells were preincubated in phenol red-free MEM supplemented with 5% CS-FBS for 48 h followed by serum-free phenol red-free MEM for 24 h. Cells were returned to phenol red-free MEM and 5% CS-FBS with or without 10 nM 17-β-estradiol. After 24 h cells were trypsinized and fixed in 100% ethanol overnight. Fixed cells were stained with Guava Cell Cycle Reagent (Millipore) and cell cycle was analyzed in a Guava Easy Cyte Mini Base System (Millipore) using the supplied statistical software exactly as described previously [16]. The data represent the mean and SE of at least three independent experiments. Statistically significant differences between data sets were determined using paired Student's *t* test.

2.8. xCELLigence cell migration assay

Cell migration was determined using the xCELLigence System (Roche) with the CIM-Plate 16 and RTCA DP Instrument (Roche) according to the manufacturer's instructions. Briefly, Cells were preincubated in phenol red-free MEM supplemented with 5% CS-FBS for 48 h and then 40,000 cells were added to the upper CIM-Plate 16 chamber in phenol red-free MEM supplemented with 0.2% CS-FBS with or without 10 nM 17-β-estradiol. Phenol red-free MEM supplemented with 5% CS-FBS with or without 10 nM 17-β-estradiol was added to the lower chamber and the CIM-Plate 16 was incubated in the RTCA DP Instrument for 48 h. Cell migration as a function of real-time changes in electrical impedance was monitored by the xCELLigence System. Cell Index (referred to here as Migration Index) and standard deviation of replicates were calculated using the supplied RTCA Software (Roche). Each sample was prepared in triplicate and the data represent the mean and SE of at least three independent experiments. Statistically significant differences between data sets were determined using paired Student's *t* test.

3. Results and discussion

3.1. HER4/4ICD is an obligate coactivator of estrogen regulated genes

We have previously shown that the HER4 intracellular domain (4ICD) coactivates and interacts with ER at the promoters of progesterone receptor (PgR) and CXCL12 (also referred to as SDF-1), but 4ICD failed to coactivate the estrogen regulated gene TFF1 (also referred to as pS2) [9,10]. These observations suggest that 4ICD is a selective ER coactivator. To determine the extent of 4ICD coactivation of β-estradiol regulated genes we developed a MCF-7 cell line with stable knockdown of HER4 (MCF-7/H4Si4) using a HER4 targeting shRNA expression construct (Fig. S1). We

first identified β-estradiol regulated genes in the vector control, MCF-7/pLKO, cell line by gene expression array. β-estradiol treatment at 100 pM for 6 h significantly stimulated 726 MCF-7/pLKO genes by 2-fold or greater and significantly suppressed 53 genes by 2-fold or greater (Table S1). To determine the extent of 4ICD coregulation of the estrogen regulated genes we performed microarray gene expression analysis of the MCF-7/H4Si4 cell line also treated with 100 pM of β-estradiol for 6 h. Loss of 4ICD expression failed to significantly alter expression of the 53 MCF-7/pLKO β-estradiol suppressed genes. These results indicate that 4ICD does not function as a β-estradiol-activated ER corepressor in MCF-7 cells. However, 11 β-estradiol regulated genes were significantly repressed by 2-fold or more in the absence of 4ICD (ZKSCAN5, DDX58, BRWD1, ADCK2, ARF1, ZNF148, PRKAA1, HKR1, ZNF627, PRPF48, PAN3) suggesting that 4ICD expression prevents transcriptional repression of these genes in response to β-estradiol (Table S2). Consistent with 4ICD functioning as an important ER coactivator, expression of 277 or 38% of the 726 MCF-7/pLKO estrogen regulated genes was significantly reduced by 2-fold or greater in the absence of 4ICD expression (Table S2). Interestingly, only eight 4ICD coregulated genes exhibited a β-estradiol response of 2-fold or greater in the absence of 4ICD (CXCL12, CYP1B1, FOS, SGK3, MYBL1, FRMD3, NUDC1, DDX21) (Table S2). Accordingly, the remaining 269 4ICD coregulated genes failed to maintain a significant response to estrogen in the absence of 4ICD, indicating that 4ICD is an obligate coactivator for 37% of the MCF-7/pLKO β-estradiol stimulated genes. Consistent with our previously published results CXCL12 was identified as a 4ICD coactivated gene in the gene array analysis (Table S2) [10]. Another previously identified 4ICD coactivated gene, PgR [9,10], was not represented on the array. We used qRT-PCR to validate 4ICD coactivation of CXCL12 and PgR but not RASGRP1 and confirm that loss of 4ICD results in enhanced TFF1 expression (Fig. 1). Consistent with our previously published results [9,10] and the microarray data presented here (Tables S1 and S2) β-estradiol stimulated TFF1 expression increased in the absence of 4ICD whereas β-estradiol stimulated expression of CXCL12 and PgR was suppressed by greater than 2 fold in the absence of 4ICD (Fig. 1). Taken together these results further establish 4ICD as a potent ER coactivator and implicate
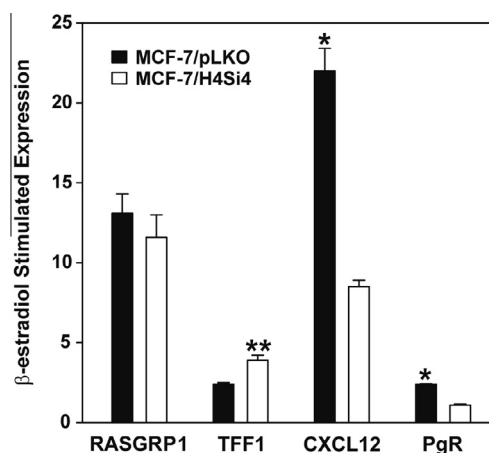
Fig. 1. Quantitative RT-PCR validation of microarray gene expression data. MCF-7/pLKO vector control and MCF-7/H4Si4 HER4 knockdown cell lines were preincubated in phenol red-free MEM supplemented with 5% CS-FBS for 48 h and were left untreated or treated with 100 pM 17-β-estradiol for 6 h. RNA was isolated and expression of the indicated genes was determined by qRT-PCR. The data represents fold change in expression of 17-β-estradiol treated versus untreated. Each sample was prepared in triplicate and the data represent the mean ± SE of at least three independent experiments. Statistically significant differences between data sets were determined using paired Student's *t* test. Asterisk and double asterisks represent $p < 0.001$ and $p = 0.011$, respectively.

4ICD as an obligate coactivator of 37% of the β-estradiol stimulated genes in breast tumor cells.

3.2. HER4/4ICD coactivates estrogen stimulated genes that function in cell cycle and tumor cell proliferation

Although 4ICD coactivates a significant percentage of β-estradiol regulated genes, it remained unclear if the 4ICD coactivated genes are associated with specific β-estradiol regulated functions or if 4ICD is a broad-spectrum ER coactivator lacking obvious functional specificity. To determine if 4ICD coactivated genes are enriched for specific β-estradiol regulated functions we performed Ingenuity Pathway Analysis (IPA) and determined the extent of functional overlap between β-estradiol regulated genes and 4ICD coactivated genes. IPA software analyzes gene expression data and predicts functional relationships within the gene set. For each of the more than 1000 IPA cellular functions an activation z-Score is calculated. An activation z-Score greater than 2 indicates a significantly increased cellular function whereas a z-Score less than -2 indicates a significantly decreased cellular function.

β-estradiol stimulation of the MCF-7/pLKO cell line was significantly associated with 36 IPA cellular functions (Table 1). Consistent with the role of β-estradiol driving breast tumor proliferation, the most significant z-Scores associated with β-estradiol regulated gene expression included multiple cellular functions associated with cellular growth and proliferation and cell cycle progression, as well as, decreased hypoplasia (Table 1). Impor-

tantly, these same functional categories had the most significant z-Scores associated with 4ICD coactivated genes (Table 2). Nevertheless, 4ICD coactivation of ER appears to have functional specificity. Accordingly, 4ICD coactivated genes fail to contribute to several significantly important β-estradiol regulated functional categories including cellular movement associated with tumor cell migration, cell death and survival associated with apoptotic resistance of tumor cells, small molecule biochemistry, gene expression related to increased transcription, and cancer associated cell transformation and metastasis (compare Tables 1 and 2).

3.3. HER4/4ICD expression is required for estrogen stimulated growth of MCF-7 breast tumor cells

We next determined if there was a concordance between the influence of 4ICD coactivation on IPA functional predictions based upon gene expression profiling and the impact of 4ICD expression on MCF-7 cells in biological assays. The IPA analysis predicted a role for 4ICD in β-estradiol stimulated MCF-7 tumor cell proliferation and cell cycle progression. To confirm these results we compared β-estradiol stimulated growth of MCF-7/pLKO cells to MCF-7/H4Si4 cells in a colony formation assay, as well as, a real-time xCELLigence growth assay. As predicted β-estradiol significantly enhanced the growth of the vector control MCF-7/pLKO cell line by 1.7-fold and 1.6-fold in the colony formation and xCELLigence assays, respectively (Fig. 2A). In contrast, the MCF-7/H4Si4 cell line failed to respond to the β-estradiol proliferative signal

Table 1
Ingenuity Pathway Analysis reveals cellular functions significantly associated with estrogen regulated genes in breast tumor cells.

Category	Functions Annotation	Regulation	p-Value	Activation z-Score
Cellular growth and proliferation	Proliferation of cells	Increased	3.80E-04	5.553
	Proliferation of tumor cell lines		2.21E-04	3.422
	Proliferation of breast cell lines		1.01E-03	2.590
	Colony formation of cells		1.08E-02	2.222
	Proliferation of epithelial cells		5.96E-03	2.108
	Proliferation of breast cancer cell lines		2.98E-03	2.006
Developmental disorder	Hypoplasia	Decreased	3.70E-03	−4.931
Cellular Movement	Cell movement	Increased	7.43E-03	3.817
	Migration of tumor cell lines		1.40E-04	3.696
	Cell movement of tumor cell lines		3.12E-05	3.025
	Invasion of cells		1.02E-03	2.980
	Cell movement of stem cells		9.52E-03	2.385
	Migration of stem cells		1.13E-02	2.176
Cell cycle	Entry into interphase	Increased	3.93E-04	3.352
	Entry into S phase		1.33E-03	3.073
	S phase		1.13E-04	2.476
	Interphase		9.23E-04	2.389
Cell death and survival	Cell Viability of tumor cell lines	Increased	5.63E-04	3.112
	Apoptosis of tumor cell lines	Decreased	1.67E-05	−2.169
	Apoptosis	Decreased	1.31E-03	−2.063
Small molecule biochemistry	Synthesis of lipid	Increased	4.21E-03	2.813
	Oxidation of lipid		1.55E-04	2.505
	Uptake of amino acids		1.08E-02	2.395
	Uptake of alpha-amino acid		2.22E-03	2.191
	Beta-Oxidation of fatty acid		8.66E-04	2.190
Gene expression	Transactivation of RNA	Increased	5.57E-03	2.734
	Transactivation		5.66E-03	2.593
	Transcription		9.12E-05	2.426
	Expression of RNA		4.48E-04	2.254
	Transcription of RNA		1.44E-04	2.240
Cancer	Cell transformation	Increased	8.61E-03	2.733
	Metastasis	Increased	8.46E-03	2.291
	Benign neoplasia	Decreased	3.09E-04	−2.081
Cellular development	Differentiation of cells	Increased	2.80E-03	2.402
DNA replication, recombination, and repair	Breakage of chromosomes	Decreased	8.60E-03	−2.348
Cellular function and maintenance	Cellular homeostasis	Decreased	8.46E-03	2.025

Table 2
Ingenuity Pathway Analysis reveals cellular functions significantly associated with estrogen regulated genes coactivated by 4ICD in breast tumor cells.

Category	Functions Annotation	Regulation	*p*-Value	Activation *z*-Score
Developmental disorder	Hypoplasia	Decreased	1.64E-03	−4.067
Cellular growth and proliferation	Proliferation of cells	Increased	1.35E-02	3.371
	Proliferation of tumor cell lines		3.88E-04	2.526
Cellular assembly and organization	Formation of cellular protrusions	Increased	1.76E-02	2.911
	Organization of cytoskeleton		2.58E-02	2.671
	Organization of cytoplasm		1.64E-02	2.671
Molecular transport	Transport of molecule	Increased	1.42E-03	2.635
Cell cycle	Entry into S phase	Increased	3.37E-03	2.592
	Interphase		9.29E-03	2.112
	S phase		6.18E-05	2.076
Energy production	Consumption of oxygen	Decreased	9.58E-03	−2.415
DNA replication, recombination, and repair	Repair of DNA	Increased	1.03E-02	2.211

with an insignificant 1.1 and 1.0-fold change in the colony formation and xCELLigence assays, respectively (Fig. 2A). These results indicate that HER4/4ICD is an obligate mediator of breast tumor cell proliferation in response to β-estradiol. To determine if 4ICD contributes to β-estradiol stimulated cell cycle progression we performed a cell cycle analysis of β-estradiol treated MCF-7/pLKO and MCF-7/H4Si4 cells. In direct concordance with the IPA functional analysis results β-estradiol stimulated cell cycle progression of the MCF-7/pLKO cell line with a significant decrease in G1 phase cells and concomitant significant increase in the percentage of S phase cells (Fig. 2B). In contrast, β-estradiol failed to stimulate cell cycle progression of the MCF-7/H4Si4 cell line (Fig. 2B). Taken together, our biological data confirms the IPA functional data underscoring the obligate role for 4ICD coactivated gene expression in β-estradiol stimulated tumor cell proliferation and cell cycle progression.

Following cellular growth and proliferation, cellular movement associated with migration of tumor cell lines was the second most significant β-estradiol stimulated MCF-7/pLKO IPA functional category (Table 1). In contrast, IPA failed to identify a significant association of 4ICD coactivated genes with the cellular movement functional category, suggesting that 4ICD coactivation does not contribute to β-estradiol-stimulated tumor cell migration (Table 2). To determine the impact of 4ICD loss-of-function on β-estradiol stimulated tumor cell migration we performed an xCELLigence cell migration assay comparing β-estradiol stimulated cell migration between MCF-7/pLKO and MCF-7/H4Si4 cell lines. Consistent with the IPA functional analysis, β-estradiol stimulated a modest but significant increase in MCF-7/pLKO tumor cell migration (Fig. 2C). Likewise, as predicted by the IPA functional analysis, suppression of HER4 expression in the MCF-7/H4Si4 cell line failed to impact β-estradiol stimulated cell migration which was equivalent to the MCF-7/pLKO cell line (Fig. 2C). Taken together, the IPA functional analysis and multiple biological assays indicate that 4ICD is a functionally selective ER coregulator primarily coactivating genes promoting β-estradiol stimulated tumor cell proliferation and cell cycle progression while failing to coactivate genes contributing to β-estradiol stimulated tumor cell migration.

Estrogen regulated gene expression and breast tumor cell growth assays have also been performed where the SRC family of canonical ER coregulators are independently suppressed. Of the three SRC family members, SRC-2 and SRC-3 (amplified in breast cancer 1; AIB1) displayed activities similar to 4ICD by contributing to estrogen stimulated growth [2,3] and cell cycle progression [2]
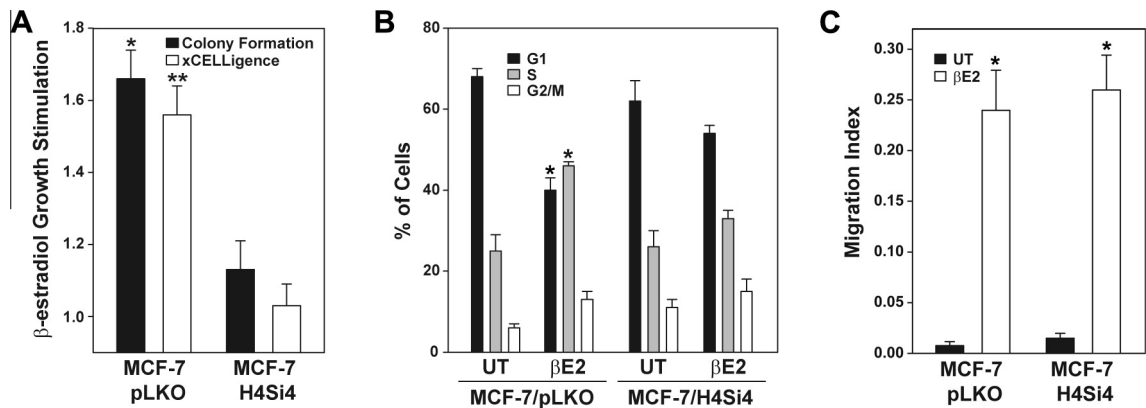
Fig. 2. HER4 expression is required for estrogen-stimulated proliferation of breast tumor cells. (A) For colony formation assay MCF-7/pLKO and MCF-7/H4Si4 cells were plated at 1000 cells per well in a 6-well plate with phenol red-free MEM supplemented with 5% CS-FBS with or without 10 nM 17-β-estradiol. Media was changed every 2 days and after 12 days the number of fixed and stained colonies was calculated using a ColCount Colony Counter. For the xCELLigence cell growth assay 2000 cells were plated in an E-Plate 16 in the RTCA DP Instrument. After 24 h media was changed to phenol red-free MEM supplemented with 5% CS-FBS and after an additional 48 h cells were left untreated or treated with 10 nM 17-β-estradiol. The slope of the change in cell index during a 72 h treatment was used to determine relative growth stimulation. Data represents 17-β-estradiol stimulated growth relative to untreated cells. (B) Cell cycle analysis of each cell line preincubated in phenol red-free MEM supplemented with 5% CS-FBS for 48 h followed by serum-free phenol red-free MEM for 24 h. Cells were returned to phenol red-free MEM and 5% CS-FBS with (βE2) or without (UT) 10 nM 17-β-estradiol for 24 h and cell cycle analysis was performed on fixed and stained cells in a Guava Easy Cyte Mini Base System. (C) Cell migration assay of each cell line preincubated in phenol red-free MEM supplemented with 5% CS-FBS for 48 h and then transferred to the upper CIM-Plate 16 chamber. The upper and lower chambers contained phenol red-free MEM with 0.2% and 5% CS-FBS, respectively, with or without 10 nM 17-β-estradiol. Using the xCELLigence System cell index (referred to here as Migration Index) and standard deviation of replicates were calculated after 48 h using the RTCA Software. For all experiments samples were prepared in triplicate and the data represents the mean ± SE of at least three independent experiments. Statistically significant differences between data sets were determined using paired Student's *t* test. Asterisk and double asterisks in (A) represent *p* = 0.013 and *p* = 0.008, respectively. Asterisk in (B) and (C) represent *p* = 0.001 and *p* < 0.005, respectively.

of MCF-7 cells. In addition, both 4ICD and SRC-3 are required for estrogen stimulated PgR expression [2,9,10]. Although there appears to be partial overlap between 4ICD and SRC family coregulated genes, 4ICD has unique activities. For example, all three SRCs bind with ER at the TFF1 promoter and independent suppression of each SRC results in suppressed estrogen induced TFF1 expression [2,17]. In contrast, 4ICD fails to bind with ER at the TFF1 promoter [10] and we consistently observe enhanced estrogen stimulated TFF1 expression when 4ICD expression is suppressed [10]. These results indicate that 4ICD fails to coactivate TFF1 and may function as an indirect TFF1 corepressor. Conversely, 4ICD is required for maximal estrogen induced MYC expression (Table S2); whereas none of the SRC family members appear to influence MYC expression [2]. These observations suggest that although 4ICD and SRC family members have some functional overlap, 4ICD exhibits coregulator activities distinct from SRC family members.

In conclusion, our data provides the first experimental evidence that a cell surface receptor directly functions as an important nuclear receptor transcriptional coactivator. Specifically, we have previously shown that 4ICD coregulates estrogen gene expression by selectively associating with ER at estrogen responsive promoters [9,10]. Here we used a global gene expression microarray approach to show that 4ICD is an obligate coactivator for 37% of the estrogen regulated genes in breast tumor cells. Furthermore, our biological and *in situ* data indicates that 4ICD selectively coactivates estrogen regulated genes associated with tumor cell proliferation and cell cycle progression. Clinically, nearly 90% of ER positive breast tumors coexpress HER4 [15] suggesting a predominant tumor cell selection for ER/4ICD coregulated gene expression driving tumor cell proliferation. By extension, our evidence that 4ICD coactivation activity is required for estrogen stimulated breast tumor proliferation implies that the majority of breast cancer patients would benefit from a therapeutic strategy that disengages ER and 4ICD cooperative signaling. Indeed, we have shown that tamoxifen mediated disruption of the ER and 4ICD transcriptional complex results in breast tumor cell apoptosis [18]. Therefore, we predict that a HER4 inhibitor, such as lapatinib, would improve patient response to endocrine therapy.

Acknowledgments

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.2013.11.111.

References

[1] V.J. Bardou, G. Arpino, R.M. Elledge, C.K. Osborne, G.M. Clark, Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases, J. Clin. Oncol. 21 (2003) 1973–1979.

[2] S. Karmakar, E.A. Foster, C.L. Smith, Unique roles of p160 coactivators for regulation of breast cancer cell proliferation and estrogen receptor-alpha transcriptional activity, Endocrinology 150 (2009) 1588–1596.

[3] H.J. List, K.J. Lauritsen, R. Reiter, C. Powers, A. Wellstein, A.T. Riegel, Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells, J. Biol. Chem. 276 (2001) 23763–23768.

[4] A.K. Shiau, D. Barstad, P.M. Loria, L. Cheng, P.J. Kushner, D.A. Agard, G.L. Greene, The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen, Cell 95 (1998) 927–937.

[5] R.L. Sutherland, R.E. Hall, I.W. Taylor, Cell proliferation kinetics of MCF-7 human mammary carcinoma cells in culture and effects of tamoxifen on exponentially growing and plateau-phase cells, Cancer Res. 43 (1983) 3998–4006.

[6] D.M. Lonard, B.W. O'Malley, Nuclear receptor coregulators: modulators of pathology and therapeutic targets, Nat. Rev. Endocrinol. 8 (2012) 598–604.

[7] G.A. Vidal, A. Naresh, L. Marrero, F.E. Jones, Presenilin-dependent γ-secretase processing regulates multiple ERBB4/HER4 activities, J. Biol. Chem. 280 (2005) 19777–19783.

[8] C.C. Williams, J.G. Allison, G.A. Vidal, M.E. Burow, B.S. Beckman, L. Marrero, F.E. Jones, The ERBB4/HER4 receptor tyrosine kinase regulates gene expression by functioning as a STAT5A nuclear chaperone, J. Cell Biol. 167 (2004) 469–478.

[9] J. Rokicki, P.M. Das, J.M. Giltnane, O. Wansbury, D.L. Rimm, B.A. Howard, F.E. Jones, The ERα coactivator, HER4/4ICD, regulates progesterone receptor expression in normal and malignant breast epithelium, Mol. Cancer 9 (2010) 150.

[10] Y. Zhu, L.L. Sullivan, S.S. Nair, C.C. Williams, A. Pandey, L. Marrero, R.K. Vadlamudi, F.E. Jones, Coregulation of estrogen receptor by estrogen-inducible ERBB4/HER4 establishes a growth promoting autocrine signal in breast cancer, Cancer Res. 66 (2006) 7991–7998.

[11] C.K. Tang, D.J. Goldstein, J. Payne, F. Czubayko, M. Alimandi, L.-M. Wang, J.H. Pierce, M.E. Lippman, ErbB-4 ribozymes abolish neuregulin-induced mitogenesis, Cancer Res. 58 (1998) 3415–3422.

[12] C.K. Tang, X.-Z.W. Concepcion, M. Milan, X. Gong, E. Montgomery, M.E. Lippman, Ribozyme-mediated down-regulation of ErbB-4 in estrogen receptor-positive breast cancer cells inhibits proliferation both *in vitro* and *in vivo*, Cancer Res. 59 (1999) 5315–5322.

[13] U.M. Domanska, R.C. Kruizinga, W.B. Nagengast, H. Timmer-Bosscha, G. Huls, E.G. de Vries, A.M. Walenkamp, A review on CXCR4/CXCL12 axis in oncology: no place to hide, Eur. J. Cancer 49 (2013) 219–230.

[14] M. Sundvall, K. Iljin, S. Kilpinen, H. Sara, O.P. Kallioniemi, K. Elenius, Role of ErbB4 in breast cancer, J. Mammary Gland Biol. Neoplasia 13 (2008) 259–268.

[15] Z. Suo, H.S. Berner, B. Risberg, M.G. Karlsson, J.M. Nesland, Estrogen receptor-alpha and C-ERBB-4 expression in breast carcinomas, Virchows Arch. 439 (2001) 62–69.

[16] D. Mitra, P.M. Das, F.E. Jones, JUMONJI/ARID1 B (JARID1B) promotes breast tumor cell cycle progression through epigenetic repression of micro RNA let-7e, J. Biol. Chem. 286 (2010) 40531–40535.

[17] P. Labhart, S. Karmakar, E.M. Salicru, B.S. Egan, V. Alexiadis, B.W. O'Malley, C.L. Smith, Identification of target genes in breast cancer cells directly regulated by the SRC-3/AIB1 coactivator, Proc. Natl. Acad. Sci. USA 102 (2005) 1339–1344.

[18] A. Naresh, A.D. Thor, S.M. Edgerton, K.C. Torkko, R. Kumar, F.E. Jones, The HER4/4ICD estrogen receptor coactivator and BH3-only protein is an effector of tamoxifen-induced apoptosis, Cancer Res. 68 (2008) 6387–6395.