Brazilian Journal of Medical and Biological Research (2008) 41: 579-582
ISSN 0100-879X Short Communication

Transcriptional up-regulation of *PHLDA1* by 17ß-estradiol in MCF-7 breast cancer cells

A.C. Marchiori, D.A. Casolari and M.A. Nagai

Disciplina de Oncologia, Departamento de Radiologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil

Correspondence to: M.A. Nagai, Disciplina de Oncologia, Departamento de Radiologia, FM-USP, Av. Dr. Arnaldo, 455, 4° andar, 01246-903 São Paulo, SP, Brasil
Fax: +55-11-3069-7292. E-mail: nagai@usp.br

Most breast cancer risk factors are associated with prolonged exposure of the mammary gland to high levels of estrogens. The actions of estrogens are predominantly mediated by two receptors, ERα and ERß, which act as transcription factors binding with high affinity to estrogen response elements in the promoter region of target genes. However, most target genes do not contain the consensus estrogen response elements, but rather degenerated palindromic sequences showing one or more mutations and other ER-binding sites such as AP-1 and SP-1. Using the differential display reverse transcription-polymerase chain reaction technique, our group identified several genes differentially expressed in normal tissue and in ER-positive and ER-negative primary breast tumors. One of the genes shown to be down-regulated in breast tumors compared to normal breast tissue was the *PHLDA1* (Pleckstrin homology-like domain, family A, member 1). In the present study, we investigated the potential of *PHLDA1* to be regulated by estrogen via ER in MCF-7 breast cancer cells. The promoter region of *PHLDA1* shows an imperfect palindrome, an AP-1- and three SP-1-binding sites potentially regulated by estrogens. We also assessed the effects of 17ß-estradiol on *PHLDA1* mRNA expression in MCF-7 breast cancer cells. MCF-7 cells exposed to 10 nM 17ß-estradiol showed more than 2-fold increased expression of the *PHLDA1* transcripts compared to control cells ($P = 0.05$). The anti-estrogen ICI 182,780 (1 µM) inhibited *PHLDA1* mRNA expression and completely abolished the effect of 10 nM 17ß-estradiol on *PHLDA1* expression ($P < 0.05$), suggesting that *PHLDA1* is regulated by estrogen via ER.

Key words: Breast cancer; Gene expression; Estrogen receptor; Estrogen response elements; *PHLDA1*

Research supported by FAPESP (#2004/04607-8 and #2006/01026-0) and CNPq (#304949/2006-0).

Received January 11, 2008. Accepted June 11, 2008

Most breast cancer risk factors are associated with prolonged exposure of the mammary gland to high levels of estrogen (E2) (1). The biological effects of estrogen are predominantly mediated by two distinct receptors, ERα and ERß, which are ligand-activated transcription factors (2). The estrogen receptors have similar structures, showing a high degree of homology in the DNA-binding domain, the most conserved region that contains two zinc finger motifs (3). Estrogen receptors bind with high affinity to estrogen response elements (EREs) in the promoter region of target genes, interact with the basal transcription machinery and regulate gene expression. The consensus ERE is a perfect palindromic DNA motif consisting of two inverted sequences of 5 bp separated by three nucleotides, 5'-GGTCAnnn TGACC-3' (4). In the human genome, however, most promoter regions of estrogen target genes do not contain the consensus ERE, but contain degenerate palindromic sequences showing one or more mutations, ERE half-sites, direct repeats of half-palindromes and other transcription factor-binding sites such as AP-1, SP-1, NF-κB, C/EBP, FoxA1, and octamers (4-6). ER also interacts with EREs containing variation in a single base, although some nucleotide substitutions can be more harmful than others (7). Some symmetrical substitutions on each side of the palindrome may more dramatically affect the interaction with the ER than a unilateral replacement (7).

The presence of the ER and progesterone receptor in breast tumors has been used as a prognostic indicator and predictive factor of response to adjuvant hormonal therapy. However, a subgroup of ER-positive breast cancer

patients either does not respond to hormonal therapy, or becomes resistant to it (8). Over the last few years, different molecular technologies that allow high throughput analysis of gene expression profiling have been used to identify the gene expression signature associated with the hormone-dependence of breast cancer that might improve our understanding of ER-positive breast cancers and help select therapy for individual patients.

Using the differential display reverse transcription-polymerase chain reaction technique, our group identified several genes differentially expressed in primary breast tumors regarding the presence or absence of ER and progesterone receptor, and a set of genes differentially expressed in normal and tumor tissues regardless ER status (9). One of the genes found to be down-regulated in breast tumors compared to normal breast tissue was the *PHLDA1* (Pleckstrin homology-like domain, family A, member 1). The *PHLDA1* gene is located on chromosome 12q15-21.2 and encodes a protein of 262 amino acids that is co-localized with *FAK* (10). The *PHLDA1* protein contains protein-protein interaction domains in its carboxy-terminal region, such as proline-histidine and proline-glutamine repeats, which may have a role in transcriptional activation and may mediate apoptosis in T cell hybridomas and in neuronal and melanoma cells (11-13).

Recently, using immunohistochemistry in tissue microarray containing a large series of samples, we demonstrated that *PHLDA1* expression is frequently down-regulated in primary breast tumors and has a predictive prognostic value (14). In the present study, we have investigated the potential regulation of *PHLDA1* by estrogen via ER in MCF-7 breast cancer cells.

To assess the potential regulation of *PHLDA1* by estradiol via ER, we performed a manual search of potential ER-binding sites in the promoter region of the *PHLDA1* gene. The NCBI (www.ncbi.nlm.nih.gov) and USCU Genome Bioinformatics (genome.uscs.edu) databases were used to annotate the position of *PHLDA1* in the genome and to extract a 5' flanking region from 2 kb upstream to 2 kb downstream of the transcription start site or transcription

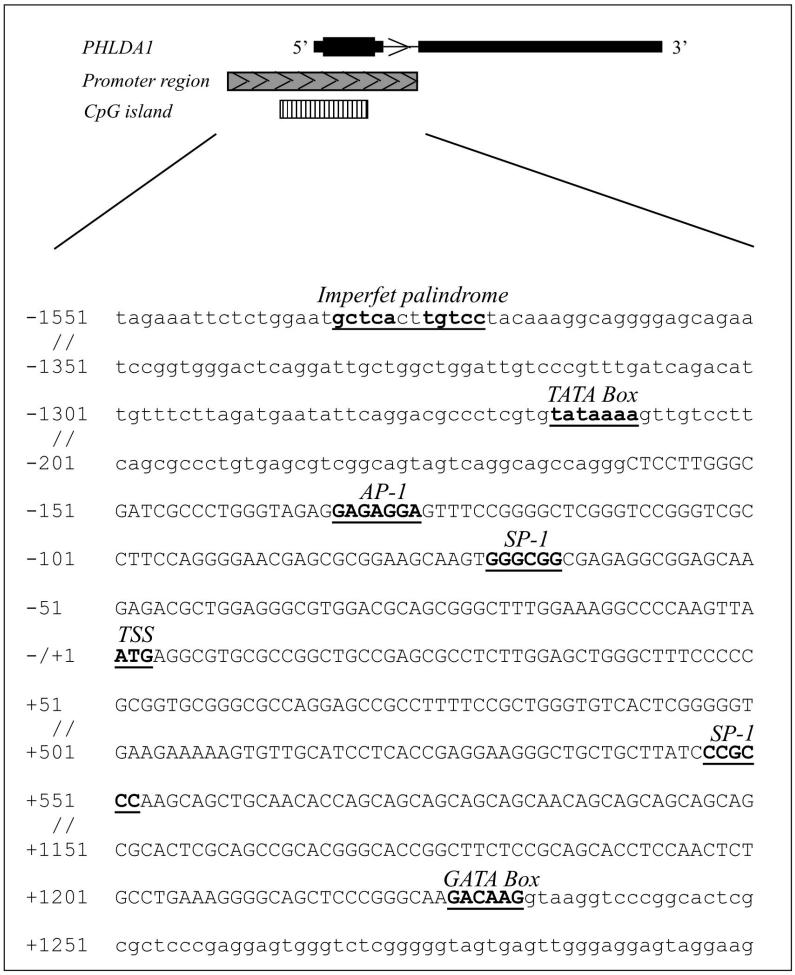

Figure 1. Structure of the human *PHLDA1* gene located on chromosome 12q15 and its partial promoter region (sequences 2 kb upstream to 2 kb downstream of the transcription start site, TSS). The numbers shown on the left indicate the nucleotide position relative to the transcription start site (+1). The sequence of the *PHLDA1* partial promoter region was obtained from www.ensembl.org. The sequences of the ATG start codon, imperfect palindrome, AP-1- and SP-1-binding sites and TATA and GATA boxes are underlined and in bold. The exons are represented in capital letters and the introns in the lower case letters.

binding site. The search for EREs and other transcription factor-binding sites was performed using several publicly available programs (Transfac 6.0, www.gene-regulation.com) and Dragon Genome Explorer (research.i2.a-star.edusg/promoter). We identified the presence of an imperfect palindrome with two mutations separated by two nucleotides at position -1522 (GCTCACTTGTCC) and direct repetitions of half-EREs and half-palindromes spread in the promoter region. Furthermore, the analysis revealed the presence of an AP-1 site at -127 (GAGAGGA), three SP-1 sites at -66 (GGGCGG), +547 (CCGCCC), +1743 (CCGCCC), a TATA box at -1261 (TATAAAA), and a GATA-1 box at +1227 (GACAAG) (Figure 1). Next we examined the effects of 17ß-estradiol and ICI 182,780 on *PHLDA1* mRNA expression in MCF-7 cells, a hormone-responsive breast cancer cell line. The MCF-7 cell line was acquired from the American Type Culture Collection (USA) and cultured as described previously (15). For the experiments, the cells were maintained in RPMI-1640 modified medium, without phenol red, and supplemented with 5% fetal bovine serum or 5% stripped fetal bovine serum, free of steroids, for 48 h before the treatments. MCF-7 cells were treated with 10 nM 17ß-estradiol (Sigma Chemical Corporation, USA) or 1 µM ICI 182,780 (TOCRIS Biosciences) alone or in combination for 2, 6, and 24 h. Anti-estrogens, such as tamoxifen (selective estrogen modulators) and ICI 182,780 (selective estrogen receptor down-regulators), are used to investigate whether or not the estrogen actions are mediated by ER pathways. Since tamoxifen also acts as an estrogen agonist in some tissues, here we used ICI 182,780 (fulvestrant), considered to be a pure estrogen antagonist, which binds to ER leading to its destabilization and degradation (16). The concentrations of 17ß-estradiol (10 nM) and ICI 182,780 (1 µM) used in our experiments were the same as used in several previous reports (17). The experiments were performed in triplicate. At each time point of drug treatment, cells were harvested and total RNA was isolated using the Illustra RNAspin kit (GE Healthcare, UK). cDNA synthesis was carried out using the High Capacity Archive kit (Applied Biosystems, UK). Real time PCR was performed using the SYBR Green kit (Applied Biosystems, USA) following manufacturer recommendations. Real time PCR products were processed with the GeneAmp 5700 Sequence Detector (PE Applied Biosystems, USA) as described previously (14). The Student *t*-test was used for statistical analysis and $P \leq 0.05$ was considered to be statistically significant.

Most of the human estrogen target genes identified so far do not contain a perfect palindromic consensus sequence in their promoter regions (4). However, several lines of evidence indicate that sequences of ERE half-sites associated with SP-1 sites, direct repeats of ERE half-sites, and EREs with different spacing between the palindromic sequences also display estrogen responses (18,19). Our analysis of the *PHLDA1* promoter region revealed the presence of several transcription factor binding sites including one imperfect palindrome, direct repeats of half-EREs, one AP1- and three SP1-binding sites, suggesting that estrogen might play a role in the transcriptional regulation of this gene. As shown in Figure 2, increased expression of *PHLDA1* transcripts (more than 2-fold) was observed in MCF-7 cells exposed to 10 nM 17ß-estradiol compared to control cells ($P = 0.05$). *PHLDA1* mRNA expression showed higher induction after 6 h of estradiol treatment. The pure anti-estrogen ICI 182,780 at 1 µM inhibited *PHLDA1* mRNA expression and completely abolished the effects of 10 nM 17ß-estradiol on *PHLDA1* expression ($P < 0.05$). These findings provide evidence that *PHLDA1* is regulated by estrogens and indicate that the

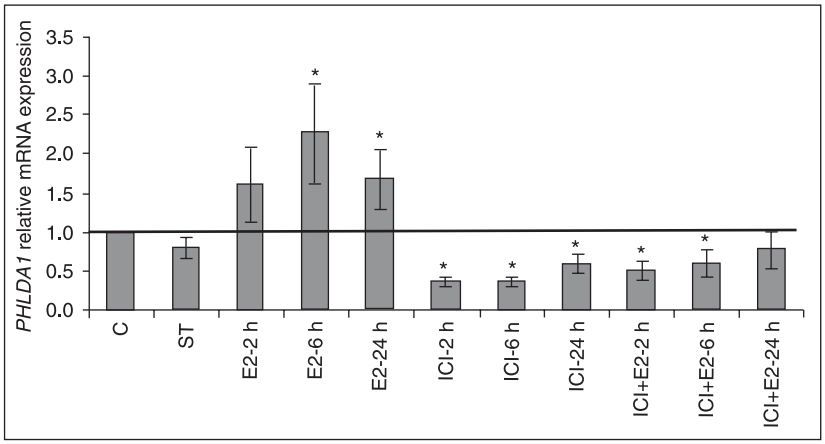

Figure 2. Effects of 17ß-estradiol and ICI 182,780 on *PHLDA1* expression in MCF-7 breast cancer cells. MCF-7 cells were treated with 10 nM 17ß-estradiol and/or 1 µM ICI 182,780 for 2, 6, and 24 h. C = cells maintained in normal serum; ST = cells maintained in treated serum; E2 = cells maintained in ST and containing 10 nM 17ß-estradiol; ICI = cells maintained in ST and containing 1 µM ICI 182,780; ICI + E2 = cells maintained in ST and containing 1 µM ICI for 1 h prior to 10 nM E2 addition. The height of the columns indicate mean ± SEM of three independent experiments. *$P \leq 0.05$ compared to cells maintained in ST (Student *t*-test). RNA expression value = 1 indicates no difference of mRNA expression, values <1 indicate down-regulation of mRNA expression and values >1 indicate up-regulation of mRNA expression.

mechanism of its regulation is ER-dependent.

Programmed cell death, or apoptosis, is a very well-controlled process, which is critical to maintain tissue homeostasis and to prevent the accumulation of damaged cells. Defects in apoptosis play important roles in tumor pathogenesis, allowing neoplastic cells, as well as genetically unstable cells, to survive (20). Constitutive *PHLDA1* expression was associated with reduced cell growth and colony formation, and increased basal apoptosis in the 293 melanoma cell line (13). Neef and co-workers (13) also showed that down-regulation of *PHLDA1* expression is associated with the progression of malignant melanomas. *PHLDA1* transcripts were also identified as being down-regulated in primary breast tumors compared to normal breast tissue (9). More recently, using immunohistochemistry in tissue microarray containing a large series of samples, we demonstrated that *PHLDA1* expression is frequently down-regulated in primary breast tumors and that the expression of *PHLDA1* irrespective of ER positivity is a marker of good prognosis in primary breast tumors (14). The estrogens act via their receptors stimulating cell growth and differentiation in the mammary gland. Although estrogens promote cell proliferation, their effect on *PHLDA1* expression might be important for the process of breast differentiation and might protect normal breast cells from uncontrolled proliferation.

Many genes are regulated by estrogens, and the investigation of how these genes are regulated and their role in breast cancer development may improve the identification of new markers that in the future may benefit patients who are under hormone therapy. Although further clinical and experimental studies are required to better understand the role played by *PHLDA1* in normal mammary glands and in breast cancer, our data demonstrated that *PHLDA1* is an estrogen target gene.

References

1. Russo IH, Russo J. Role of hormones in mammary cancer initiation and progression. *J Mammary Gland Biol Neoplasia* 1998; 3: 49-61.
2. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, et al. Mechanisms of estrogen action. *Physiol Rev* 2001; 81: 1535-1565.
3. Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ER-alpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* 1999; 140: 5566-5578.
4. Gruber CJ, Gruber DM, Gruber IM, Wieser F, Huber JC. Anatomy of the estrogen response element. *Trends Endocrinol Metab* 2004; 15: 73-78.
5. Kushner PJ, Agard D, Feng WJ, Lopez G, Schiau A, Uht R, et al. Oestrogen receptor function at classical and alternative response elements. *Novartis Foundation Symposium*. Chichester, New York: Wiley; 2000; 230: 20-26.
6. Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, et al. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* 2005; 122: 33-43.
7. Bourdeau V, Deschenes J, Metivier R, Nagai Y, Nguyen D, Bretschneider N, et al. Genome-wide identification of high-affinity estrogen response elements in human and mouse. *Mol Endocrinol* 2004; 18: 1411-1427.
8. Ring A, Dowsett M. Mechanisms of tamoxifen resistance. *Endocr Relat Cancer* 2004; 11: 643-658.
9. Nagai MA, Ros N, Bessa SA, Mourao NM, Miracca EC, Brentani MM. Differentially expressed genes and estrogen receptor status in breast cancer. *Int J Oncol* 2003; 23: 1425-1430.
10. Kuske MD, Johnson JP. Assignment of the human PHLDA1 gene to chromosome 12q15 by radiation hybrid mapping. *Cytogenet Cell Genet* 2000; 89: 1.
11. Park CG, Lee SY, Kandala G, Lee SY, Choi Y. A novel gene product that couples TCR signaling to Fas(CD95) expression in activation-induced cell death. *Immunity* 1996; 4: 583-591.
12. Gomes I, Xiong W, Miki T, Rosner MR. A proline- and glutamine-rich protein promotes apoptosis in neuronal cells. *J Neurochem* 1999; 73: 612-622.
13. Neef R, Kuske MA, Prols E, Johnson JP. Identification of the human PHLDA1/TDAG51 gene: down-regulation in metastatic melanoma contributes to apoptosis resistance and growth deregulation. *Cancer Res* 2002; 62: 5920-5929.
14. Nagai MA, Fregnani JH, Netto MM, Brentani MM, Soares FA. Down-regulation of PHLDA1 gene expression is associated with breast cancer progression. *Breast Cancer Res Treat* 2007; 106: 49-56.
15. De Bessa SA, Salaorni S, Patrao DF, Neto MM, Brentani MM, Nagai MA. JDP1 (DNAJC12/Hsp40) expression in breast cancer and its association with estrogen receptor status. *Int J Mol Med* 2006; 17: 363-367.
16. Lewis JS, Jordan VC. Selective estrogen receptor modulators (SERMs): mechanisms of anticarcinogenesis and drug resistance. *Mutat Res* 2005; 591: 247-263.
17. Nagai MA, Brentani MM. Gene expression profiles in breast cancer to identify estrogen receptor target genes. *Mini Rev Med Chem* 2008; 8: 448-454.
18. Anderson I, Gorski J. Estrogen receptor alpha interaction with estrogen response element half-sites from the rat prolactin gene. *Biochemistry* 2000; 39: 3842-3847.
19. O'Lone R, Frith MC, Karlsson EK, Hansen U. Genomic targets of nuclear estrogen receptors. *Mol Endocrinol* 2004; 18: 1859-1875.
20. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007; 35: 495-516.