

***CTCF* and *CTCFL* mRNA expression in 17 β -estradiol-treated MCF7 cells**

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Abstract. Estrogens play a key role in breast cancer, with 60-70% of the cases expressing estrogen receptors (ERs), which are encoded by the *ESR1* gene. CTCFL, a paralogue of the chromatin organizer CTCF, is a potential biomarker of breast cancer, but its expression in this disease is currently controversial. A positive correlation has been reported between CTCFL and ERs in breast tumors and there also exists a coordinated interaction between CTCF and ERs in breast cancer cells. Therefore, there appears to be an association between CTCF, CTCFL and estrogens in breast cancer; however, there has been no report on the effects of estrogens on *CTCF* and *CTCFL* expression. The aim of this study was to determine the effect of 17 β -estradiol (E2) on the *CTCF* and *CTCFL* mRNA expression in the MCF7 breast cancer cell line. The promoter methylation status of *CTCFL* and data mining for estrogen response elements in promoters of the *CTCF* and *CTCFL* genes were also determined. The transcription of *CTCF* and *CTCFL* was performed by quantitative polymerase chain reaction (qPCR) and the promoter methylation status of *CTCFL* was determined by methylation-specific PCR. The MCF7 cells exhibited basal transcription of *CTCF*, which was significantly downregulated to 0.68 by 1 μ M E2; basal or E2-regulated transcription of *CTCFL* was not detected. Under basal conditions, the *CTCFL* promoter was methylated. Through data mining, an estrogen response element was identified in the *CTCF* promoter, but no such element was found in *CTCFL*. These results suggested that estrogens may modulate *CTCF* expression, although there was no apparent association between ERs and *CTCFL*.

Introduction

Breast cancer is the leading cause of mortality among women worldwide, although the underlying molecular mechanisms have not been fully elucidated. Estrogens are crucial in breast cancer, with 60-70% of the cases expressing estrogen receptors (ERs), predominantly the α -ER, which is encoded by the *ESR1* gene (1,2). Therefore, the elucidation of the mechanisms underlying the effect of estrogens on breast cancer is of paramount importance.

The *CTCFL* gene, encoding the CTCFL protein, also referred to as BORIS (Brother of the Regulator of Imprinting Sites), has recently emerged as a potential biomarker of female breast cancer, as it is normally expressed only by male germ cells. In a previous study, it was demonstrated that the *CTCFL* gene is expressed in malignant and non-malignant breast cell lines, as well as in ~70% of the clinical specimens of breast cancer, but not in normal breast tissue (3). *CTCFL* is a paralogue of *CTCF*, the gene encoding CTCF, a ubiquitous 11-zinc finger protein with highly versatile functions, such as the global three-dimensional genome organization, including intra- and interchromosomal loop formation (4-8). CTCF is involved in transcriptional silencing or activation and may function as an insulator and chromatin organizer. CTCF shares the 11-zinc finger protein region with CTCFL and the co-expression of the two genes was previously demonstrated in breast cancer (9). However, the expression of *CTCFL* in breast cancer is currently highly controversial. Since the first report of its expression in the majority of clinical breast specimens (3), subsequent studies were highly divergent, with results ranging from complete absence of *CTCFL* expression in breast cancer (10), to its ubiquitous expression in normal and malignant tissues (11).

A positive correlation between the levels of CTCFL and ER in breast tumors was previously described (3), suggesting that CTCFL may be under estrogen regulation. In addition, there exists a coordinated interaction between CTCF and ER in breast cancer cells, as CTCF binding to DNA co-localizes with ER sites (12,13); it is hypothesized that in these sites of

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co-localization, CTCF may mark the euchromatic regions, allowing ER to bind and activate or repress the expression of target genes. Therefore, a pro-transcriptional role was suggested for CTCF in ER-mediated gene expression in breast cancer cells (13). CTCF and CTCFL appear to be associated with estrogens and ER in breast cancer; however, the knowledge on this subject is currently scant.

The role of estrogens on the regulation of *CTCF* and *CTCFL* gene expression has not yet been investigated and its determination may help elucidate the biology of breast cancer. Therefore, the aim of this study was to investigate the effect of 17 β -estradiol (E2) on the *CTCF* and *CTCFL* mRNA expression in MCF7 breast cancer cells, which represent a suited model for the *in vitro* study of estrogenic pathways, as they express ERs.

Materials and methods

Cells. The MCF7 (HTB-22) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). This cell line was derived from the breast adenocarcinoma of a Caucasian female and was shown to express ERs. The MCF7 cells also express high CTCF levels (14).

Cell culture conditions. Immediately following their acquisition, the MCF7 cells were propagated by culture in 60-mm polystyrene dishes with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), amphotericin and gentamycin, at 37°C in a 5% CO₂/95% air atmosphere. In order to determine the estrogenic effect on *CTCF* and *CTCFL* transcription, the MCF7 cell cultures at high density were incubated for 24 h in DMEM, with 0.2% human albumin instead of FBS. Subsequently, the cells were incubated for 20 h with E2 (1,3,5-estratriene-3,17 β -diol; Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 0.01, 0.1 and 1 μ M. At the end of the incubation period, total RNA was obtained with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quantitation and purity were determined by spectrophotometry in a Beckman Coulter DU730 apparatus (Beckman Coulter Inc., Fullerton, CA, USA) at an absorbance of 260 nm and an absorbance ratio of 260/280 nm, respectively. The final product was stored at -40°C until use in quantitative reverse transcription polymerase chain reaction (qRT-PCR) within the following 3 days.

The E2 was prepared as a 1x10⁻³ M stock solution in absolute ethanol. The controls included ethanol, which in previous experiments did not exert any effect on the expression of the genes under investigation. The experiments were performed in triplicate and repeated three times per biological replica.

qPCR for *CTCF* and *CTCFL* mRNA expression. First, cDNA was synthesized in 20- μ l volume reactions from 1 μ g total RNA with the Verso cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). The obtained cDNA (1 μ l) was then amplified in 15- μ l volume reactions with gene-specific primers and probes of the Solaris qPCR Gene Expression Assay system (Thermo Fisher Scientific) according to the manufacturer's recommendations. *GAPDH* was used as a reference gene due to its good performance, as previously reported (15). The

Table I. Effect of E2 on *CTCF* transcription in MCF7 cells.

E2 (μ M)	Relative expression	95% CI	P-value
0.01	0.834	0.588-1.177	0.201
0.1	0.830	0.368-1.662	0.177
1	0.684	0.343-0.857	0.000

E2, 17 β -estradiol; CI, confidence interval.

primers had consensus sequences that recognized all the splice variants of the genes under investigation and they all exhibited identical temperature conditions for amplification. qPCR was performed in a Eco thermocycler (Illumina, San Diego, CA, USA) under the following conditions: a 15-min step at 95°C to activate polymerase, followed by 40 cycles at 95°C for DNA denaturation and 60°C for annealing-extension. The efficiency of the reactions was 99, 95 and 101% for *GAPDH*, *CTCF* and *CTCFL*, respectively. A formalin-fixed paraffin-embedded specimen of breast cancer expressing *CTCFL* was used to determine the efficiency and specificity of the amplification of this gene, as its expression in MCF7 cells was not detected under any conditions, as described below.

Statistical analysis. The comparative data were analyzed with REST 2009 software (Qiagen GmbH, Hilden, Germany) employing 6,000 randomizations. P<0.05 was considered to indicate a statistically significant difference.

Methylation analysis of the *CTCFL* promoter. Genomic DNA (1 μ g) was bisulfite-modified with the Imprint DNA Modification kit (MOD50; Sigma-Aldrich) and eluted in a final volume of 20 μ l, following the manufacturer's protocol. The modified DNA was stored at -40°C and used within 1 week. Methylation-specific PCR (MSP) for the *CTCFL* promoter was performed with 1 μ l of the modified DNA in a final reaction volume of 25 μ l, containing 12.5 μ l of GoTaq Master Mix (Promega Inc., Madison, WI, USA), 0.5 μ l of each forward and reverse primers described elsewhere (16), and 10.5 μ l of water.

The PCR amplification consisted of 35 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 45 sec. The amplification products were analyzed by agarose gel electrophoresis with ethidium bromide staining.

Estrogen response elements in *CTCF* and *CTCFL* promoters. Data mining for localization of the ER binding sites in the human *CTCF* and *CTCFL* promoter was performed using the online software LASAGNA-Search, developed by the Department of Computer Science and Engineering, University of Connecticut, Storrs, CT (17) available at http://biogrid.engr.uconn.edu/lasagna_search/.

Results

Effect of E2 on *CTCF* and *CTCFL* mRNA expression. In order to investigate the effects of E2 on the mRNA transcription of

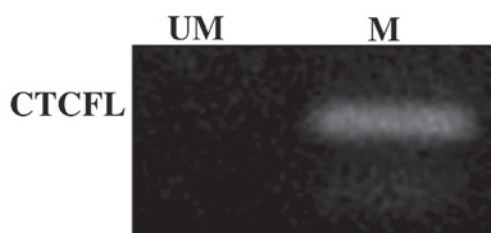


Figure 1. Methylation status of the *CTCFL* promoter, qualitative MSP analysis. Left, amplification products for unmethylated (UM) promoter; right, amplification products for methylated (M) promoter. Only the methylated amplicon is observed.

CTCF and *CTCFL*, qRT-PCR was performed. The MCF7 cells exhibited a basal mRNA transcription as expected. E2 exerted a statistically significant downregulating effect to 0.68 the value of the control. E2 at concentrations of 0.01 and 0.01 μ M also exerted a downregulating effect, although it was of no statistical significance (Table I).

By contrast, basal transcription of *CTCFL* in MCF7 cells was not detected and incubation with E2 did not result in *CTCFL* mRNA upregulation under any hormone concentration. Therefore, the possible downregulating effects of E2 on *CTCFL* expression could not be assessed.

Analysis of methylation of the *CTCFL* promoter. To further investigate the underlying mechanisms responsible for the lack of expression of the *CTCFL* gene, the methylation state of its promoter was determined by MSP under basal conditions and only the methylated promoter was detected (Fig. 1), which was suggestive of gene silencing.

Data mining for estrogen response elements in the *CTCF* and *CTCFL* promoters. As estrogen response elements have not been described in the *CTCF* and *CTCFL* promoters, a search for consensus sequences was performed with the LASAGNA-Search software. Only a consensus sequence for *CTCF* was identified in the minus strand, 247 bp upstream from the transcription start site; this sequence was GTCCGCTTGACCT. By contrast, consensus sequences for *CTCFL* were not identified.

Discussion

ER are the driving transcription factors in the majority of breast cancers; when coupled to estrogens they activate or inhibit genes involved in cell cycle progression and cell survival during malignant transformation. CTCF is linked to the ER biology through interactions that have not yet been fully elucidated. CTCFL is a CTCF paralogue, which is expressed in breast cancer. To date, a role for estrogens in the regulation of CTCF and CTCFL expression has not been reported. In this study, the effect of E2 on the transcription of the *CTCF* and *CTCFL* genes in the MCF7 breast cancer cell line was investigated.

We did not detect any basal *CTCFL* transcription in MCF7 cells. There are discordant reports regarding *CTCFL* expression in this cell line; Hines *et al* (10) and Vatolin *et al* (18) did not detect any *CTCFL* expression by conventional RT-PCR

and/or qPCR methods. By contrast, Renaud *et al* (19) reported *CTCFL* expression in MCF7 cells measured by qPCR and northern blot analysis. In addition, D'Arcy *et al* (3) detected *CTCFL* expression by RT-PCR, western blot analysis and immunostaining. The inconsistency between those studies may be attributed to the heteroclonal nature of MCF7 cells; this cell line was established in 1973 and MCF7 cells have since been widely distributed worldwide, resulting in different stocks exhibiting clonal heterogeneity (20). In the studies that were in agreement with the present study, the source of MCF7 cells was not specified by Vatolin *et al* (18), who mentioned that some of the cell lines they used were obtained from ATTC, whereas Hines *et al* (10) mentioned that the cell lines were obtained from ATCC. As regards studies in disagreement with the present study, D'Arcy *et al* (3) acknowledge M. O'Hare and B. Gusterson for providing breast cell lines, whereas Renaud *et al* (19) did not mention the source. Therefore, the clonal heterogeneity of the MCF7 cells appears to be a plausible explanation for the divergence in *CTCFL* expression results.

Basal expression of *CTCF* was found as expected, according to previously reported findings (14). CTCF is a ubiquitously expressed regulator of fundamental cellular events, including transcription, intra- and interchromosomal interactions and chromatin structure (7). The ENCODE project (21) unveiled the significance of CTCF in long-range chromatin interactions. A total of 77,811 distinct binding sites for CTCF were identified across 19 cell types, which underlines the importance of this factor in maintaining genome integrity (22), although up- and downregulation of its expression by diverse stimuli have been reported; for example, *CTCF* overexpression has been associated with resistance to apoptosis in breast cancer cell lines (14) and its downregulation has been reported in epithelial ovarian cancer (23). In this study, a downregulating effect on *CTCF* transcription by E2 was documented; thus, further studies are required to investigate whether this hormone modulates *CTCF* transcription *in vivo*, directly or indirectly.

The results of the promoter methylation analysis of *CTCFL* are in agreement with the results obtained from the gene transcription analysis depicted above, as the methylation of the *CTCFL* promoter is indicative of gene silencing.

Furthermore, the data mining for consensus sequences of estrogen response elements in the promoters was in agreement with the results, as only a sequence for *CTCF* was identified, suggesting that it is a target for ERs, unlike *CTCFL*. However, distant estrogen response elements in the genes and long-range interactions, as well as indirect effects of E2 on the two genes, cannot be excluded.

In conclusion, this study demonstrated that E2 downregulated *CTCF* mRNA expression in MCF7 cells, which did not exhibit basal transcription of *CTCFL*, whereas E2 did not exert any upregulating effects on *CTCFL* mRNA. These results suggest that there is an independent association between ER positivity and *CTCFL* expression in breast cancer. However, further investigations on this subject are required.

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