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Estrogen induced concentration dependent differential gene expression in human breast cancer (MCF7) cells: Role of transcription factors

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ABSTRACT

Background: Breast cancer cells respond to estrogen in a concentration dependent fashion, resulting in proliferation or apoptosis. The mechanism of this concentration dependent differential outcome is not well understood yet.
Methodology: Meta-analysis of the expression data of MCF7 cells treated with low (1 nM) or high (100 nM) dose of estradiol (E2) was performed. We identified genes differentially expressed at the low or the high dose, and examined the nature of regulatory elements in the vicinity of these genes. Specifically, we looked for the difference in the presence, abundance and spatial distribution of binding sites for estrogen receptor (ER) and selected transcription factors (TFs) in the genomic region up to 25 kb upstream and downstream from the transcription start site (TSS) of these genes.
Results: It was observed that at high dose E2 induced the expression of stress responsive genes, while at low dose, genes involved in cell cycle were induced. We found that the occurrence of transcription factor binding regions (TFBRs) for certain factors such as Sp1 and SREBP1 were higher on regulatory regions of genes expressed at low dose. At high concentration of E2, genes with a higher frequency of Oct-1 binding regions were predominantly involved. In addition, there were differences in the spatial distribution pattern of the TFBRs in the genomic regions among the two sets of genes.
Discussion: E2 induced predominantly proliferative/metabolic response at low concentrations; but at high concentration, stress–rescue responses were induced. At high E2 concentration, classical genomic pathway involving ER binding to the regulatory regions was reduced, and alternate or indirect activation of genes through Oct-1 became more prominent.

1. Introduction

Estrogens are an important class of hormones in the physiology of mammals, regulating cell proliferation, differentiation and apoptosis [1,2]. Cellular effects of estrogens are predominantly brought about by their interaction with estrogen receptors (ER). Upon ligand binding, the ERs in the cytoplasm dissociate from the heat shock proteins, dimerise and translocate to the nucleus. In the nucleus, the ligand bound ER interact directly or indirectly with specific regions of the DNA and act as a transcription factor (TF), recruit other TFs and coactivators/repressors, and thereby modulating the expression of the target genes [3]. The short stretches of DNA, where the ligand-bound ER directly binds are called as Estrogen Response Elements (EREs) [4]. In addition to the direct binding, ER can form complexes with other TFs, and direct their binding to their respective response elements on the target genes [3,4]. The regulation of a single gene can be under the control of several TFs, including ER [5,6], with the binding regions occurring close (5–10 kb) to the transcription start site (TSS), or as distal elements [7,8].

The cellular response to estrogens is closely linked to their concentration. In breast cancer cells abundant in ERs, such as MCF7, estrogens cause marked proliferative effect at picomolar concentration [9]. 10 nM is assumed be the saturation concentration of estrogen for ERs in MCF7 cells [10]. But at concentrations above 10–20 nM, estrogens direct the cells towards apoptosis [11]. An intriguing question is how the same cell responds contrastingly to different concentrations of estrogens [12].

In this study, meta-analysis of the expression data from MCF7 cells treated with 1 or 100 nM of E2 for 3 h was done. Specifically, we looked for the difference in the occurrence, abundance and distribution of binding sites for ER and other TFs in the genes which were differentially expressed in response to 1 or 100 nM E2.

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2. Methods

2.1. Datasets

Expression datasets of MCF7 cells treated with two different concentrations of E2, produced from the same set of microarray platform (GPL570, Affymetrix Human Genome U133 Plus 2.0 Array), were obtained from Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo). We selected GSE11506 [13] and GSE11324 [14] expression data of MCF7 cells treated with 1 and 100 nM E2 for 3 h, respectively. The data were downloaded through the GEO2R interface (http://www.ncbi.nlm.nih.gov/geo/geo2r/), with *P*-values adjusted by the Benjamini and Hochberg (False Discovery Rate) method. The entries in each set were sorted based on adjusted *P*-value, from the most to the leastsignificant value.

The top 1000 entries each from GSE11506 and GSE11324 were parsed to remove redundant gene-name entries and probe set entries not mapping to valid annotated RefSeq gene names. After redundancy reduction, GSE11506 gave 765 unique entries, while 774 entries remained from GSE11324. Further, unique non-overlapping genes from these two sets of non-redundant entries were used to create the 1 nM_T1 K dataset ($n = 566$) and 100 nM_T1 K dataset ($n = 595$), respectively. Similarly, we created a non-redundant, non-overlapping list of genes from the 1000 entries from rank 10,000 to 10,999 of GSE11506 and GSE11324, which were designated as 1 nM_T10 K ($n = 715$) and 100 nM_T10 K ($n = 792$), respectively.

Custom Track option in UCSC Genome Browser (http://genome.ucsc.edu/) for Human Genome Build hg19 was used to retrieve the repeat-masked, genomic sequence (TSS ± 25 kb) for each gene entry (GE) in 100 nM_T1 K, 100 nM_T1 K and 1 nM_T1 K datasets. Thus, each gene sequence entry (GSE) in the datasets was a unique, repeat-masked 50 kb (TSS ± 25 kb) genomic sequence. The workflow for the design of 1 nM_T1 K and 100 nM_T1 Kis outlined in Supplementary Fig. 1.

2.2. Software and webservers

Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/)[15] was used for mapping the Affymetrix probe set data to statistically relevant gene ontology (GO) terms and plausible cellular pathways. KEGG pathway database [16] was employed in the Functional Annotation Toolbox of DAVID to retrieve the significant cellular pathways.

MATCH v8 [17] was used for determining the TFBRs in each GSE in the datasets. TRANSFAC matrix with minimum "False Positives" option for each TF was used in MATCH. The dollar sign "$" preceding the protein name implies TFBR.

GeneMania [18] plugin for Cytoscape 2.8 was used to identify the physically interacting TFs among the 59 TFs reported by Lin et al., [19] found to be enriched in ER binding genomic regions. The network weights were assigned based on gene ontology –"Biological Process".

3. Results and discussion

3.1. Unique sets of genes were induced by E2 in a dose dependent manner

To determine the difference, if any, in the cellular response of MCF7 cells exposed to low (1 nM) or high (100 nM) dose of E2, DAVID was used for mapping the top 1000 Affymetrix probe set ids from GSE11506 and GSE11324 to relevant GO terms and to probable KEGG pathways.

From the top 1000 probe set entries of GSE11506 and GSE11324, DAVID assigned 348 and 167 "Biological Process" GO terms, respectively (Fig. 1). Of these, approximately 60% ($n = 241$) GO terms were unique to low dosage induction and 15% ($n = 60$) to high dosage, while only 25% ($n = 107$) were common to both the sets. Thus, at the low dose, the cellular processes were more diverse when compared to high dose.

In MCF7 cells treated with the high dose of E2, a large number of the responding genes mapped to ontology terms associated with "response to stress" and "cellular response to stress". These ontology terms were conspicuously absent in low dose responsive condition (see Supplementary Table 1). These results suggested that predominantly different process pathways were triggered by E2 at low and high doses. Also, high concentration of E2 induced stress–rescue in cells while inducing diverse proliferative/metabolic responses at lower concentration. Several gene expression studies report that E2 at concentrations below 25 nM can induce

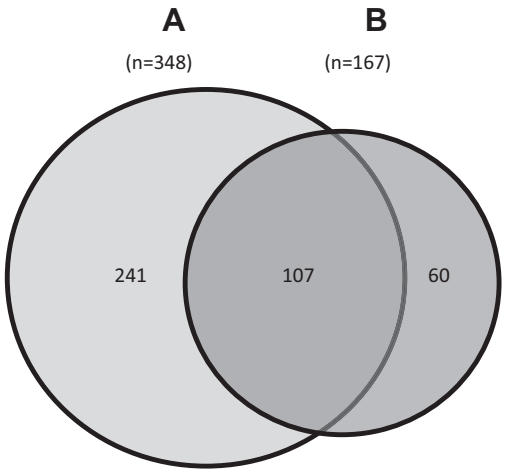

Fig. 1. Venn diagram (not to scale) showing the number of unique and overlapping GO terms (n) mapped from top 1000 probe ids of GSE11506 (A) and of GSE11324 (B) at 3 h of E2 treatment. The full list of GO terms from this analysis is available in Supplementary Table 1.

Table 1
The key KEGG pathways to which the top 1000 probeset entries of GSE11506 and GSE11324 were mapped by DAVID. *P*-value represents the level of significance from Fisher's Exact Test. The details of the genes mapped (Gene counts) in each pathway is available in Supplementary Table 2. MAPK pathway and p53 pathway annotated with the mapped genes are available in Supplementary Figure 1.

Dataset	Pathway	Gene counts	*P*-value	KEGG pathway ID
GSM11506	Colorectal cancer	13	1.8×10^{-4}	hsa05210
	Acute myeloid leukemia	10	6.3×10^{-4}	hsa05221
	Pathways in cancer	27	1.2×10^{-3}	hsa05200
	Regulation of actin cytoskeleton	19	3.9×10^{-3}	hsa04810
	Chronic myeloid leukemia	10	4.0×10^{-3}	hsa05220
	MAPK signaling	22	4.0×10^{-3}	hsa04010
	Endometrial cancer	8	5.7×10^{-3}	hsa05213
	Fc gamma R-mediated phagocytosis	11	6.4×10^{-3}	hsa04666
	ErbB signaling	10	1.0×10^{-2}	hsa04012
GSM11324	p53 Signaling	9	2.5×10^{-3}	hsa04115
	Pathways in cancer	23	2.5×10^{-3}	hsa05200
	Colorectal cancer	10	2.6×10^{-3}	hsa05210
	Pyrimidine metabolism	10	6.1×10^{-3}	hsa00240

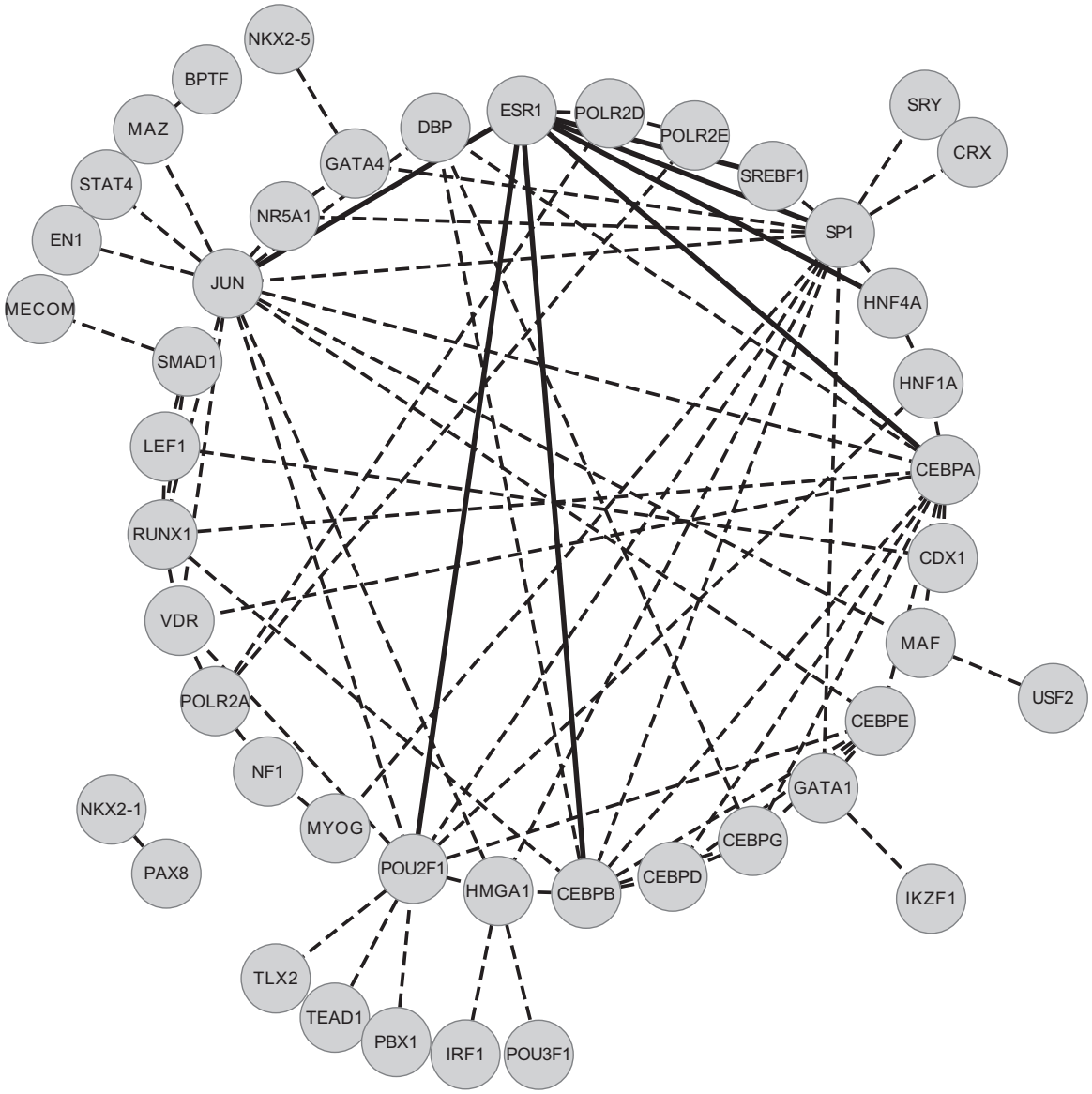

Fig. 2. Physical interaction network map of TFs, including ESR1 and RNA Polymerase II subunits, produced by GeneMania. The interconnecting lines indicate that evidence for physical interaction between the nodes is available from the databases used by GeneMania. The interaction between ESR1 and its partner is represented by a bold unbroken line. The list of TFs and the association network is available as Supplementary Table 3.

robust expression of genes responsible for transcriptional activity and proliferation [20,21].

Significant cellular pathways ($P \leqslant 0.01$) assigned by DAVID from the top 1000 probe set entries of GSE11506 and GSE11324 are listed in Table 1. Some pathways such as "colorectal cancer" and "pathways in cancer" were common between the sets, while majority of the pathways were unique.

The pathway mapping (Table 1) was again indicative of the difference in cellular response between the low and high E2 exposure. At low E2, key proliferative signaling pathways such as MAPK signaling pathway (hsa04010) and ErbB signaling pathway (hsa04012) were active. ErbB and MAPK signaling leads to cell motility, proliferation, differentiation, and survival [22,23] along with the associated PI3K/Akt pathway [24]. At high E2 exposure, the cell response as represented by the number of GO terms and the number of functional pathways were significantly restricted (Fig. 1 and Table 1). p53 signaling pathway (hsa04115) was the significant pathway under this condition (Table 1). p53 phosphorylation is one mechanism by which cells respond to stress stimuli, and activation of this pathway could result in cell cycle arrest, senescence or apoptosis [25].

3.2. ER interacted with a limited number of TFs

Lin et al., [19] reported that the genomic fragments pulled down by ER-specific antibody in a modified ChIP-seq experiment were significantly enriched with binding sites for 59 TFs. The possible interaction of estrogen receptor alpha (ESR1) with these TFs was analyzed using GeneMania. GeneMania creates interaction networks using a variety of sources, including BioGRID and PathwayCommons [18]. The results are shown in Fig. 2. It was reported by GeneMania that ESR1 physically interacted with only seven TFs, among these TFs. These seven TFs were CEBPA, CEBPB, JUN/AP1, HNF4A, POU2F1/Oct-1, Sp1 and SREBF1/SREBP1. The RNA Polymerase II subunits (POLR2*) were included in the network to visualize the interaction(s) of TFs with the polymerase complex.

Table 2
Count of GSEs in each dataset with at least one occurrence of the respective TFBR. The number within the parenthesis represents the percentage of the GSEs with a specific TFBR in the respective dataset.

TF	1 nM_1TK [n = 566]	100 nM_1TK [n = 595]	TRANSFAC matrix ID
ER	510 (90.10%)	484 (81.34%)	$ER_Q6
ER	473 (83.56%)	436 (73.27%)	$ERALPHA_01
Sp1	444 (78.44%)	413 (69.41%)	$SP1_Q6_01
SREBP1	544 (96.11%)	559 (93.94%)	$SREBP1_Q6
Oct-1	509 (89.92%)	566 (95.12%)	$OCT1_Q6
CEBPA	556 (98.23%)	585 (98.31%)	$CEBPA_Q6
CEBPB	560 (98.93%)	587 (98.65%)	$CEBPB_Q6
HNF4A	304 (53.71%)	302 (50.75%)	$HNF4A_Q6_01
AP1	350 (61.83%)	364 (61.17%)	$AP1_Q6_02

3.3. The abundance of TFBRs were significantly different for GSEs between the datasets

MATCH was used to determine the frequency of the different TFBRs in the GSEs from each of the dataset (Table 2). It was observed that the GSEs of both the datasets had similar frequency of TFBRs for the selected TFs. However, the frequencies for ER and Sp1 binding sites were higher in the low dose induced dataset, while in high dose induced dataset, the binding sites for SREBP1, Oct-1 and CEBPA/B were seen to be higher.

Fig. 3A represents the distribution statistics of binding sites of ER and the seven ER interacting TFs in GSEs from 1 nM_T1 Kand 100 nM_T1 K Fig. 3B shows the abundance of TFBRs in the GSEs of 1 nM_T10 K and 100 nM_T10 K datasets. The distribution statistics of ERE half site ($ERALPHA_01) and androgen receptor (AR) binding site ($AR_03) are also included in the figure. $AR_03 was an "internal control" across the datasets since AR activation is not reported to occur with E2 exposure.

Interestingly, the number of GSEs with $SREBP1 was nominally higher in 100 nM_T10 K(Table 2), but its frequency of occurrence within the GSEs was significantly higher in 1 nM_T1 K(Fig. 3A). SREBP1 is a transcription factor regulating expression of genes responsible for severalmetabolic processes like fatty acid synthesis [26]. Strong co-association between SREBP1 and Sp1 have been shown in several large scale studies including the ENCODE project [27].

Frequency analysis of 100 nM_T10 Kand 1 nM_T10 Kshowed that there were no significant differences between the abundances of the TFBRs between them (Fig. 3B). The AR binding sites ($AR_03), showed no significant differences within and among the *1TK and *T10 K datasets, indicating that the observed differences in abundance of ER interacting TFBRs were non-random. Sur-

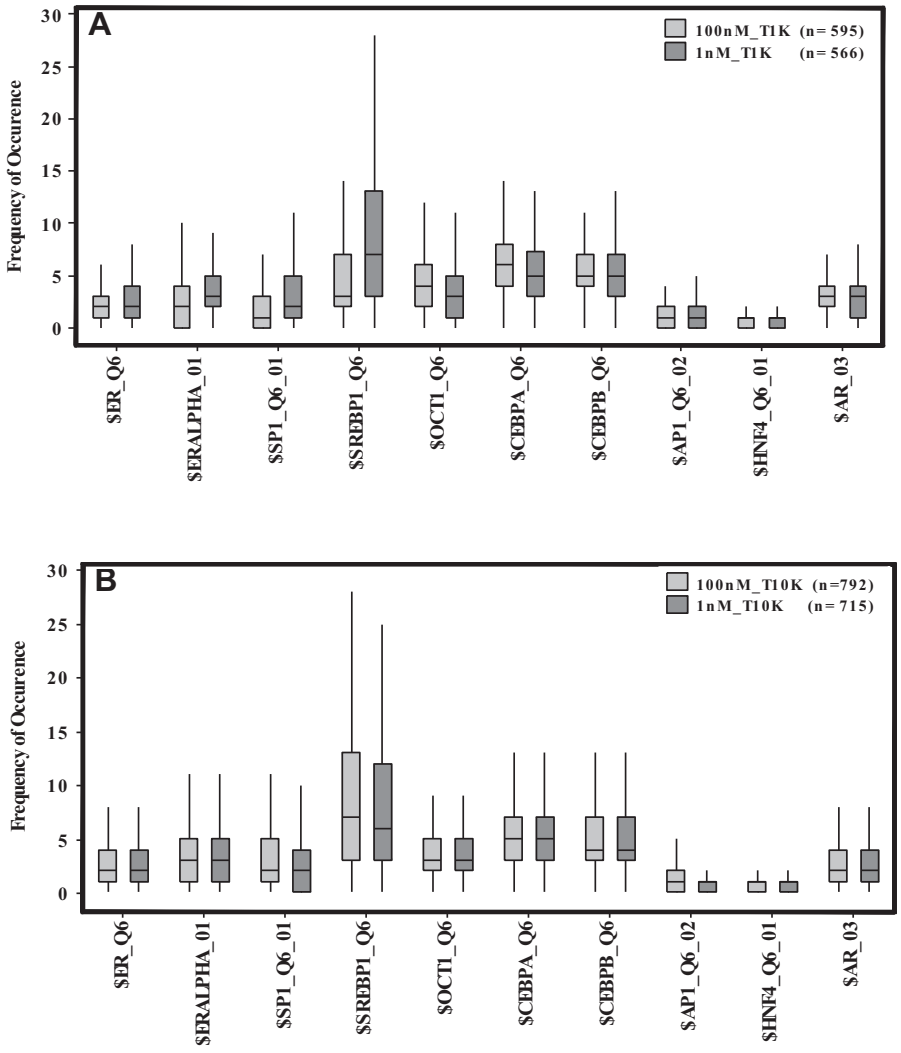

Fig. 3. A box and whiskers plot showing the frequency of occurrence of TFBRs reported to interact with ER, either directly or indirectly, and of Androgen Receptor ($AR_03) in (A) 1 nM_T1 K and 100 nM_T1 K datasets (B) 1 nM_T10 K and 100 nM_T10 K datasets. Outliers have been omitted from the chart for the sake of clarity.

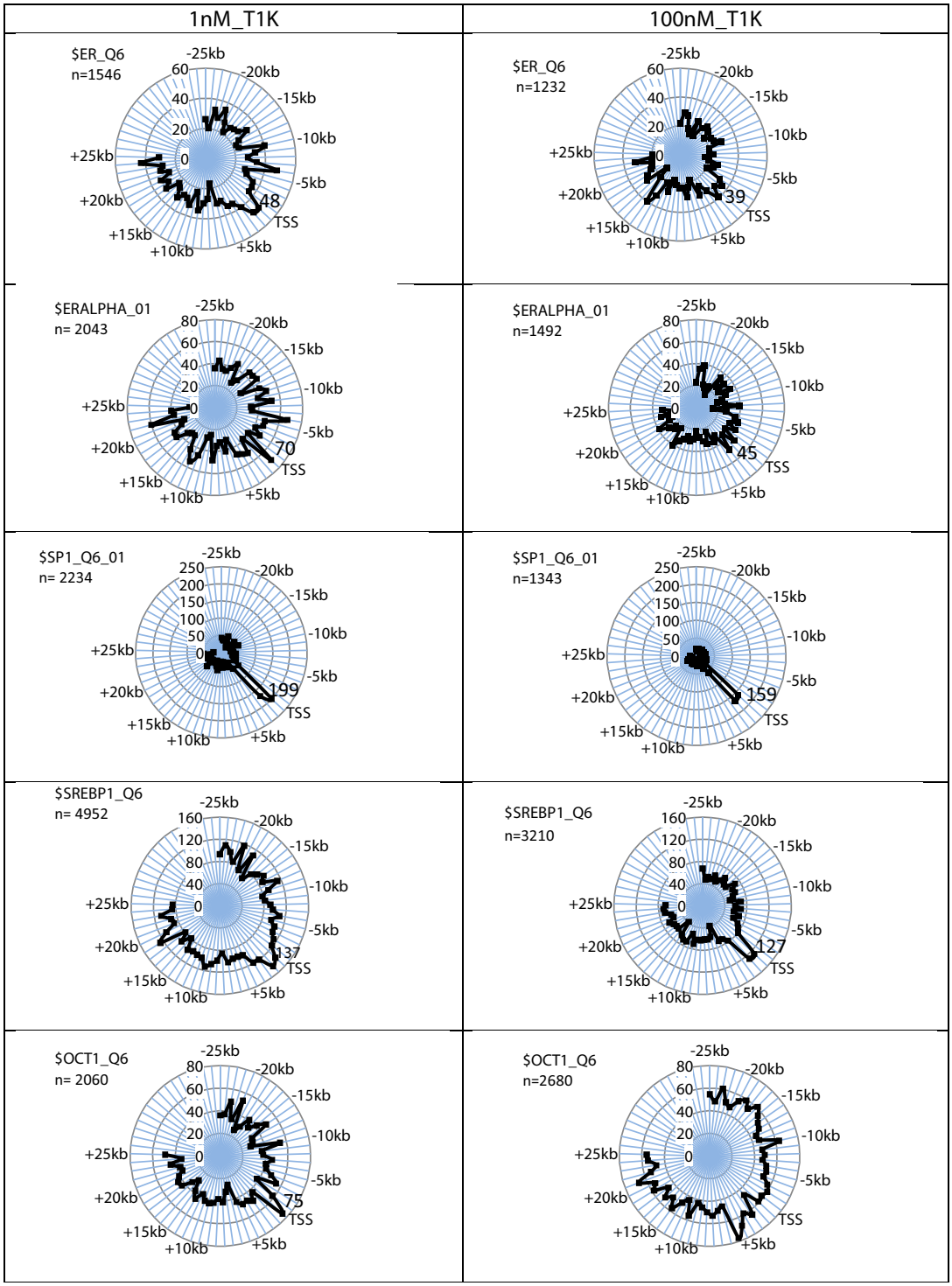

Fig. 4. Frequency distribution of TFBRs in GSEs of 1 nM_T1 K and 100 nM_T1 K datasets. The total number of individual TFBRs in each dataset is represented by "*n*".

prisingly, the number of AP1 ($AP1) and HNF4A ($HNF4A) binding sites in the GSEs were sparse. Several genome-wide analysis studieshave shown that TFs show non-random preferences for genes, often according to the functional context of the respective genes [7,14,19,28].

Fig. 3A showed that the frequency of Oct-1 and CEBPA binding sites were higher in 100 nM_T10 Kcompared to 100 nM_T10 Kdataset. ER is reported to bind and interact with Oct-1 [29]. At high E2 concentration, genes with more Oct-1 binding sites appeared to be preferentially expressed compared to low concentration exposure, which may represent an early stress–rescue response. The Oct-1/POU2F1 transcriptional activity is associated with rescue mechanisms from cellular stress [30] and repression of nuclear receptorsignaling [31] and the transcriptional activity of Oct-1 is regulated by its phosporylation state [32]. Phosphorylated Oct-1 shows altered affinity for DNA binding and transcriptional regulation [32]. Activation or deactivation of Oct-1 is mediated by activity of kinases, especially Protein Kinase A [32,33] or by cyclic AMP (cAMP) levels [34]. Several studies shows that the membrane bound estrogen receptor (GPR30) can activate or deactivate kinases [35] and alter cellular cAMP levels [36] thereby affecting the cell cycle. Therefore it is possible that estrogen can influence

the activation/deactivation ofOct-1 through GPR30 mediated signaling, although the role of estrogen concentration in this regulation is unclear yet. Members of the CCAAT/enhancer-binding proteins (CEBPs) are reported to induce cell differentiation and control cell proliferation at transcriptional level[37] or by direct inhibition of CDK2 / CDK4 [38] and may have role in some stress responses [39].

Stress response related genes in the 100 nM_T10 Kdataset with Oct-1 binding sites included ATR1, THNB1 and CASP9. Products of these genes are involved in the key p53 signaling pathway (KEGG Pathway ID: hsa04115) which produces stress–rescue responses like DNA repair, cell cycle arrest, senescence and apoptosis upon stress stimuli. ATR1 is an early stage kinase, which upon activation,phosphorylates and activates p53 protein, thus triggering a signaling cascade [40]. Interestingly, the GSE for ATR1 did not show the occurrence of either $ER or $ER_ALPHA.

3.4. The positional distribution of TFBRs showed preference for the TSS

The frequency of occurrence of individual TFBR for every 1000 base window in each GSE to determine the positional distribution of TFBRs is shown in (Fig. 4). ER binding sites ($ER_Q6 and $ERALPHA_01), were more or less randomly distributed across the 50 kb, except that their frequencies between ±5 ±10 kb from the TSS were lower in both the datasets. The observed distribution of $ER and $ER_ALPHA is consistent with the assumption that the ER binding regions can occur in the proximity of the TSS as part of the promoter elements, or can occur as distal enhancer elements [5] and therefore need not be restricted to the TSSregion.

Unlike the binding sites for ER and Sp1, the spatial distribution pattern of SREBP1 and Oct-1 binding sites showed differences between two datasets. The distribution of $SREBP1 was significantly restricted to the TSS among the GSEs of 100 nM_T1 Kwhile in 1 nM_T1 Kits distribution was high throughout and maximal near the TSS. Interestingly, the positional distribution of Oct-1 binding sites ($OCT1_Q6) was markedly different between 1 nM_T1 Kand 100 nM_T1 K. In 1 nM_T1 Kthe Oct-1 binding sites were concentrated near the TSS, while in 100 nM_1T K they were represented more throughout and concentrated near 5 kb downstream of the TSS.

In summary, the E2 different sets of genes atdifferent doses, triggering different pathways and outcome. At low concentration of E2, genes with abundant Sp1 and SREBP1 binding sites were transcriptionally active, while high concentration of E2 increased the expression of genes with predominantly Oct-1 binding sites. At high E2 concentration, regulatory pathway through ER binding on EREs was reduced, and alternate or indirect activation of genes through Oct-1 mediated regulation become more predominant. The distribution of the ER binding sites ($ER and $ER_ALPHA) occurred as part of proximal promoters (±1–5 kb) or as distant elements (±10–15 kb), while the other ER interacting TFBRs occurs in the proximity (±1–5 kb) of the TSS. The distribution pattern of $SREBP1 and $Oct-1 were markedly different between the GSEs represented at low concentration and at high concentration E2, and points to the possible differences at the regulatory level.

Inconclusion, several large scale studies like the ENCODE project [27] are underway to understand the gene regulation process at genome level [27,28]. These studies have shown that the mammalian gene regulation is a complex event involving multiple, but selective TFs [28,41]. But the role of ligand concentration in inducing differential cellular responses is poorly understood yet. Our meta-analysis indicates that ligand concentration is a significant factor in the complex process of cell response, often deciding the fate of the cell. Therefore, correlating the role of ligand concentration in gene regulation can be a significant breakthrough in understanding disease etiology and rational design of treatment regimes for diseases, including breast cancer.

Acknowledgments

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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.2013.06.108.

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