Bioinformatic analysis of changes in RNA polymerase II transcription stimulated by estradiol in MCF7 cells

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Estradiol (E2) is the most potent estrogen and RNA polymerase II (Pol II) regulates a great mass of gene expression. This study was designed to illustrate the mechanisms of estrogen-dependent human breast cancer (BC) through Pol II. ChIP-seq data, DNase-seq data and other sequencing data of human BC MCF-7 cells were downloaded from Gene Expression Omnibus database. Each of these datasets included one control and one E2 treated sample. Sequence alignment was performed and Pol II factor binding signal was determined. Functional enrichment analysis of particular genes was performed, along with transcription factor (TF) motif enrichment analysis. Sites with enhanced Pol II binding were identified in intergenic regions. Pol II binding sites with increased binding signal facilitated chromosome switching from a closed to an open state. A total of 59 TFs, including *KLF4* (Kruppel-like factor 4), were identified. Besides, enrichment analysis revealed that protein synthesis and metabolic processes as well as cell cycle processes were highlighted. The *KLF4* motif was found to be enriched and was significantly enhanced in the promoter region for Pol II binding. Cell cycle processes, protein synthesis and metabolism play critical roles in the progression of E2-stimulated BC. In addition, *KLF4* may be important for the progression.

Key words: estradiol, breast cancer, RNA polymerase II, transcription factors

Breast cancer (BC) is characterized by lumps in the breast tissue, changes in breast shape, skin dimpling, fluid secretion through the nipples, or swollen lymph nodes, bone pain, yellow skin, and shortness of breath [1]. Globally, BC affects approximately 12% of women and is the most frequent invasive cancer in women [2]. BC resulted in 458,503 deaths worldwide in 2008 [3], and it is alone expected to account for 29% of all new cancers in women in 2015[4]. Estradiol (E2), or more precisely, 17β-estradiol, is the predominant hormone secreted by the ovaries. It affects many tissues, including bone [5], and is essential for the development and maintenance of female reproductive tissues [6]. E2 is widely known to have proliferative effects on estrogen-dependent cancer cells, and antiestrogens are known to inhibit this effect.

Hormone-dependent BC, which is also known as estrogen-dependent BC, requires estrogen to sustain tumor growth. Key et al. found that [7] the risk of BC increases significantly with increasing concentrations of several hormones, including E2. Cauley et al. [8] reported that elevated serum E2 is a strong risk factor for BC. However, the precise mechanism by which E2 affects BC is complex and is controlled by multiple factors. For example, in BC cells, E2 can regulate the expression and secretion of tumor necrosis factor-α [9], which is closely linked to BC growth [10]. All cancers, which show dysregulated DNA transcription and cellular growth, are regulated by various transcription-related factors. RNA polymerase II (Pol II) is an elongation factor that synthesizes precursors of mRNAs and most small nuclear RNAs and microRNAs. It requires a range of transcription factors (TFs) for binding to promoters [11]. In addition, the Pol II core promoter and TFs are key components in the regulation of gene expression. Therefore, the process of tumor progression in E2-dependent BC is likely to be also influenced by Pol II and associated TFs.

In the current study, we measured and compared dynamic changes in the intensity of Pol II binding in human BC MCF7 cells. Based on these results, we identified candidate genes involved in the degradation process of the E2 stimulus

in MCF7 cells. Finally, using the promoter regions of these genes, transcriptional regulation elements associated with E2 stimulus were screened using TF motif enrichment analysis. This study identified key genes and biological processes involved in the proliferation of cancerous MCF7 cells stimulated by E2.

Materials and methods

Microarray datasets. ChIP-seq data (accession number: GSE23701) [12] generated using a GPL9115 Illumina Genome Analyzer II (*Homo sapiens*) platform were downloaded from the Gene Expression Omnibus (GEO) database. This dataset was collected by Joseph et al. [12], using samples with E2 stimulation or without stimulation, using the vehicle as a control. Fragments were isolated using formaldehyde-assisted isolation of regulatory elements (FAIRE) and then deep sequenced. From this dataset, we used Pol II sequence data from E2-treated MCF-7 cells (GSM588576) and non-treated (DMSO as vehicle) MCF-7 cells (GSM588577).

DNase-seq data (accession number: GSE33216) collected by He et al. [13] were also included. The selected data were from samples of MCF-7 cells treated with E2 (GSM822390) or not (GSM822389), collected using an Illumina Genome Analyzer (*Homo sapiens*) GPL9052.

Sequencing data (accession number: GSE43070) deposited into the GEO database by Jin et al. [14] were also included. Data were collected using a GPL10999 (Illumina Genome Analyzer IIx (*Homo sapiens*) or a GPL11154 (Illumina HiSeq 2000 *Homo sapiens*) platform. MCF-7 cells were treated with E2 (GSM1154040) or without reagent (GSM1154039).

Sequence alignment. The reads for the Pol II ChIP-seq and DNase-seq data were aligned against the human reference genome (UCSC hg19) using Bowtie software version 0.12.9 [15]. Only unique matches with a mismatch number ≤2 were included. Only reads that met these criteria were used for subsequent analysis.

TopHat [16], as an efficient read-mapping algorithm, was applied to align RNA-Seq reads. Default parameter settings were used, and only reads uniquely matched to the genome with ≤2 mismatches were included. Combined with reference sequence (RefSeq) gene annotation, transcript assembly and expression estimation were performed with the Cuffdiff tool in the Cufflinks package [17], based on fragments per transcript kilobase per million fragments mapped (FPKM).

Determination of Pol II factor binding sites. The Self-Administration of Medication tool (SAMtools) [18] was employed to remove potential PCR duplicates form the Pol II ChIP-seq data. When multiple reads mapped to the same locus, flattened operation was adopted to ensure that the number of reads was ≤1. With a sliding 100-bp window, modification levels were quantified by counting the number of reads (extended to 100 bp both up- and down-stream) that overlapped with a target region. The Model-based Analysis of ChIP-seq (MACS) algorithm with the MACS software

version 1.4.0 [19] was used to identify enriched regions (peak calling), with p<0.00001.

Analysis of changes in Pol II binding signal. Normalized reads per million (RPM) analysis was performed to normalize read depth and number. Differential analysis of the degree of enrichment of each peak between samples was performed with Noise Sensitivity Questionnaire (NOISeq) software, with a significance threshold of q ≥0.8 [20].

Functional enrichment analysis. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) [21] is commonly used for systematic and integrative analysis of large sets of genes and proteins. In the current study, Gene Ontology (GO) – biological process (BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted using DAVID. Default parameter settings were used.

Transcription factor (TF) motif enrichment analysis. For motif discovery, the region from 1000 bp upstream to 500 bp downstream of the transcription start site (TSS) was scanned using the Seqpos [22] algorithm. TF-enriched motifs with p<0.001 and Z-score <−10 were selected.

Results

Pol II binding sites with differential signal intensity. Based on the ChIP-seq data, 37,844 Pol II binding sites with high signal intensity were identified. A total of 7812 (20.64%) different binding sites were identified, including 5753 that showed a significant increase and 2059 that showed a significant decrease after E2 stimulus.

The distribution of E2 stimulus-related Pol II binding sites was analyzed. Binding site peaks were mainly located in the intergenic regions, for those with increased and reduced binding intensity, and those in promoter regions were the next most common.

Effect of changes in chromosome structure on Pol II binding. Figure 1 shows that no significant changes in DNase I hypersensitive site (DHS) signal were observed around the sites that showed decreased Pol II binding following E2 stimulation. Moreover, regardless of the E2 stimulus, regions around Pol II binding sites were more open than the flanking regions. However, the reduced binding ability of Pol II did not result in switching of the regional chromosome structure from an open to a closed state. In contrast, DHS signal was enhanced in the sites that showed increased Pol II binding following E2 stimulation, and the chromosome structure showed a more open state than prior to E2 stimulus. Further analysis of binding sites in promoters around the sites that showed increased Pol II binding showed significant transition to the open state.

Annotation and functional analysis of differentially binding regions. Adjacent genes were identified by annotating promoter regions that contained different Pol II binding signals. These 1727 sites with increased signal corresponded to 1696 genes including 1520 encoding genes and
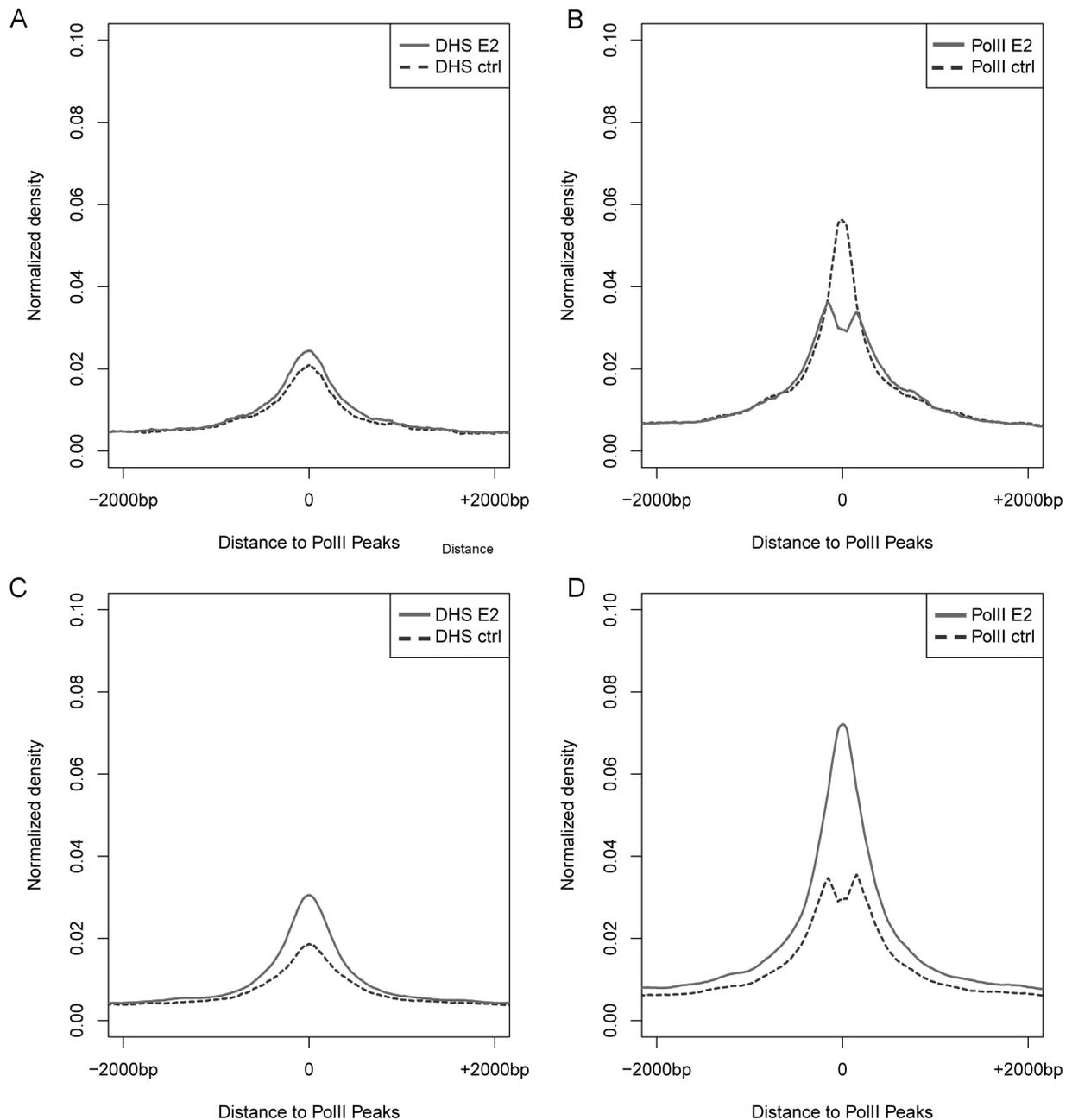
Figure 1. RNA polymerase II (Pol II) and DNase I hypersensitive site (DHS) signal near Pol II binding sites that showed differential binding affinity before and after estradiol (E2) stimulus. The upper and lower figures show the distribution of sites with reduced and increased Pol II binding after E2 stimulus, respectively. The left and right figures show the distribution of DHS and Pol II signal before and after E2 stimulus, respectively. The solid and dashed curves indicate signal before and after stimulus, respectively.

176 that encoded non-coding RNAs. In the 1520 sites that encoded genes, 59 were identified TFs. Further analyses revealed that some of these genes were tumor-related, such as *KLF4* (Kruppel-like factor 4), *MAFB* (v-maf avian musculo-aponeurotic fibrosarcoma oncogene homolog B), and *MUM1* (melanoma associated antigen 1). The 535 sites with reduced Pol II binding corresponded to 526 genes. Genes including

BC suppressor genes such as *BRCA2* (breast cancer 2, early onset) and tumor-suppressor transcription factors, *CTBP1* (C-terminal binding protein 1), *NR4A1* (nuclear receptor subfamily 4, group A, member 1), *SMARCB1* (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1), and *SOX11* (SRY, sex determining region Y – box 11) were identified. In addition,

526 genes including 470 encoding genes (17 TFs) and 56 encoding non-coding RNAs, were identified among the 535 Pol II binding sites with decreased signal.

GO enrichment analysis (Table 1) revealed that genes in the region with increased binding signal were mainly protein trafficking-related, such as protein targeting and protein transport genes. Genes in the region with decreased binding signal mainly showed cell cycle enrichment. However, this result was not significant ($p<0.01$, FDR >0.05).

KEGG pathway enrichment analysis of genes in the region with increased binding signal showed that these genes were mainly involved in ubiquitin mediated proteolysis, Fc gamma R-mediated phagocytosis, and MAPK (mitogen-activated protein kinase) signaling pathway etc. (Table 2). In particular, 11 genes were related to cell cycle pathways. No pathways were found to be associated with genes in the region with decreased binding signal.

TF motif enrichment analysis. A total of 10 significantly enriched TFs were identified in the promoter region with increased binding signal (Table 3). The identified genes included *E2F1* (E2F transcription factor 1), *EGR1* (early growth response 1), and *KLF4*, which were all cancer-related. However, *KLF4* was not only enriched in the region with increased binding signal, but was also significantly enhanced in the promoter region.

The combined expression profile data showed that only *KLF4* was up-regulated, and the other 9 TFs in the regions

Table 1. Functional enrichment analysis of RNA polymerase II binding regions responded genes.

	Term	Count	p-value	FDR
PolII E2 up	GO:0006605~protein targeting	38	1.25E-06	0.002278595
	GO:0015031~protein transport	93	1.98E-06	0.003609962
	GO:0017038~protein import	27	3.30E-06	0.006015974
	GO:0046907~intracellular transport	82	3.76E-06	0.006861461
	GO:0008104~protein localization	103	3.81E-06	0.006937147
	GO:0006913~nucleocytoplasmic transport	29	1.07E-05	0.0194838
	GO:0051169~nuclear transport	29	1.37E-05	0.024956618
	GO:0033365~protein localization in organelle	27	2.52E-05	0.045906739
PolII E2 down	GO:0007049~cell cycle	35	1.98E-04	0.334412741
	GO:0022402~cell cycle process	28	2.41E-04	0.407175908
	GO:0000279~M phase	19	5.71E-04	0.961234423

Count: the enriched gene number. GO: gene ontology. FDR: False Discovery Rate.

Table 2. Pathway enrichment analysis of increased RNA polymerase II signal sites corresponding genes.

Term	Count	p-value	FDR
hsa04120:Ubiquitin mediated proteolysis	24	8.81E-05	0.107648451
hsa04110:Cell cycle	20	0.001276895	1.549729414
hsa04666:Fc gamma R-mediated phagocytosis	13	0.037255899	37.1307681
hsa04010:MAPK signaling pathway	28	0.04051981	39.68717661
hsa00510:N-Glycan biosynthesis	8	0.043189031	41.70647181
hsa04660:T cell receptor signaling pathway	14	0.043418825	41.87737816

Count: the enriched gene number. FDR: False Discovery Rate.

Table 3. Motif enrichment analysis of transcription factor of different RNA polymerase II signal region responding genes.

	TF	Tumor Association	Enriched Motif
E2 down	SUT1	–	
E2 up	E2F1	Tumor suppressor gene	
	EGR1	Tumor suppressor gene	
	GABPA	–	
	KLF4	Oncogene	
	NFE2L1	–	
	SP2	–	
	SP4	–	
	TEAD2	–	
	TFAP2A	–	
	ZBTB7B	–	

TF: transcription factors. E2: estradiol stimulated
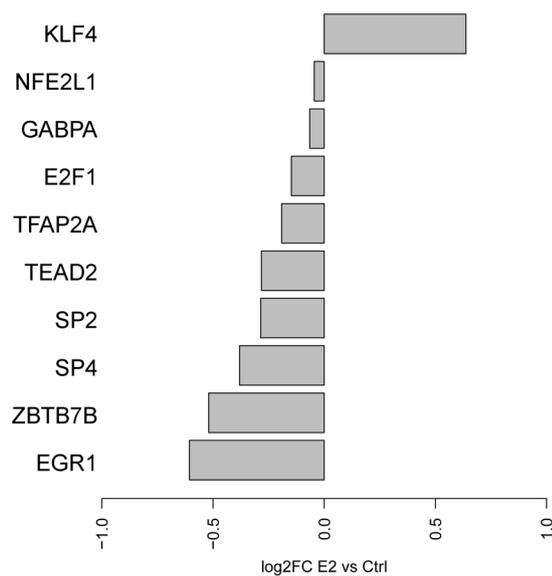
Figure 2. Changes in expression of 10 sites significantly enriched for transcription factor motifs at sites with increased RNA polymerase II signal. FC: fold change. E2: estradiol stimulated. Ctrl: control.

with increased Pol II signal were down-regulated to varying degrees (Figure 2). Out of the genes at sites with decreased Pol II signal, only *SUT1* (solute carrier family 13, sodium/sulfate symporter, member 4) showed significant TF motif enrichment.

Discussion

The mechanism underlying the development of estrogen-dependent BC is complex and remains unclear, and many further studies are needed. In the current study, three different microarray datasets (ChIP-seq, DNase-seq, and other sequencing data) collected for the human BC cell line MCF7 were analyzed. Sites with peaks of Pol II binding were mainly located in intergenic regions. A total of 59 TFs, including *KLF4* and *MAFB*, were identified. *KLF4* was TF motif-enriched and showed significantly enhanced binding signal in the promoter region of Pol II. In addition, GO and pathway enrichment analyses revealed that protein synthesis and metabolic processes, as well as cell cycle processes, may be important for the progression of E2-stimulated BC.

E2 affects gene expression by binding to E2 receptors in the cytosol, followed by translocation to the nucleus. Since it alters the Pol II binding signal, E2 affects Pol II binding efficiency and regulates expression of genes [23]. Because Pol II mainly acts on exon regions for transcription and the RNA Pol II core promoter is a key component in the regulation of gene expression [24], we chose to focus on genes transcribed by Pol II. In addition, changes in DHS signal revealed activation of gene expression, which helped to identify E2 stimulus-related transcriptional activation of genes, combined with

Pol II binding signal. The subsequent functional enrichment results suggested that functions related to protein synthesis and metabolism, including protein targeting, protein transport, and ubiquitin mediated proteolysis, were significantly enriched. Zhang et al. suggested that proteins involved in metabolic pathways are highly expressed in HER-2/neu-positive BC [25]. The role of the ubiquitin–proteasome system in neoplastic disease has been reported [26]. Therefore, E2, as the most potent form of estrogen, could contribute to the development of BC through protein transport, location, and metabolism. In addition, genes transcribed by enhanced Pol II binding were also enriched in the cell cycle pathway. These results are consistent with those reported in previous studies, which illustrated that cell proliferation is accelerated in estrogen-dependent BC [27, 28]. Thus, E2-stimulated BC is initiated by mechanisms including protein transport and metabolism and the cell cycle.

Several genes were highlighted in this study. *KLF4*, which encodes Kruppel-like factor 4, regulates the expression of key TFs during embryonic development [29]. *KLF4* expression was found to be up-regulated and corresponded to the sites with increased Pol II binding signal. In addition, *KLF4* was TF motif-enriched in sites with increased Pol II binding signal. A previous study found that *KLF4* is both an oncogene and a tumor suppressor gene [30]. In estrogen-dependent BC, *KLF4* may inhibit the transcriptional activity of estrogen receptor-alpha (ERα) and is transcriptionally activated by p53 [31]. Through regulation of the expression of manganese superoxide dismutase (MnSOD) and *KLF4*, estrogen plays an antiproliferative role in vascular smooth muscle cells [32]. A previous study demonstrated that *KLF4* inhibits the growth of estrogen-dependent BC by suppressing ERα activity [31]. GO-BP enrichment analysis showed that *KLF4* had dual regulatory functions such as negative and positive regulation of cell proliferation. These results indicate the complicated regulatory mechanism of cell proliferation. Based on the results described above, *KLF4* may play important roles in the progress of BC development following E2 stimulus.

Conclusion

Analysis of Pol II binding signal revealed changes in the expression of genes, including those related to the cell cycle as well as protein transport, location, and metabolism, which play critical roles in the progression of E2-related BC. In addition, genes such as *KLF4* that perform dual regulatory functions in processes such as cell proliferation were found to be up-regulated in E2-stimulated BC. These results may help to elucidate the complex regulatory mechanism of cell proliferation.

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