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Review

Identification of estrogen-responsive genes based on the DNA binding properties of estrogen receptors using high-throughput sequencing technology

Kazuhiro IKEDA[1], Kuniko HORIE-INOUE[1], Satoshi INOUE[1, 2, *]

[1]*Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical University, Saitama 350–1241, Japan;* [2]*Department of Anti-Aging Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo 113–8655, Japan*

Estrogens are important endocrine hormones that control physiological functions in reproductive organs, and play a pivotal role in the generation and progression of breast cancer. Therapeutic drugs including anti-estrogen and aromatase inhibitors are used to treat patients with breast cancer. The estrogen receptors, ERα and ERβ, function as hormone-dependent transcription factors that directly regulate the expression of their target genes. Therefore, a better understanding of the function and regulation of estrogen-responsive genes provides insight into the gene regulation network associated with breast cancer. Recent technological developments in high-throughput sequencing have enabled the genome-wide identification of estrogen-responsive genes. Further elucidating the estrogen gene cascade is critical for advancements in the diagnosis and treatment of breast cancer.

Keywords: steroid hormone; estrogen; nuclear receptor; gene transcription; breast cancer; high-throughput sequencing technique

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Introduction

The endocrine system produces hormones that have important roles in the development and function of various tissues. Particularly, steroid hormones exert functions through their cognate receptors that act as hormone-dependent transcription factors. Estrogen, glucocorticoid, mineralocorticoid, progesterone, and androgen bind to the estrogen receptor (ER), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), and androgen receptor (AR), respectively[1, 2]. Physiological functions of these steroid hormones are mediated through transcriptional regulation of the target genes[3]. Steroid hormone receptor-related transcription networks also contribute to various pathological states, such as cancer, osteoporosis, and inflammation[4]. Importantly, estrogen is considered to be involved in the generation and promotion of breast cancer through estrogen signaling. After the hormone binds to its receptors in a cell, it turns on hormone-responsive genes that promote DNA synthesis and cell proliferation[5]. Therefore, elucidating the function and regulation of estrogen-regulated genes would uncover the pathophysiology of breast cancer[6]. In this review, we will discuss recent insights into the role of ER signaling in breast cancer development, which is shown by data from deep-sequencing techniques, including RNA-sequencing (RNA-seq) as well as the genome-wide chromatin immunoprecipitation (ChIP) profiling analyses.

* To whom correspondence should be addressed.
E-mail INOUE-GER@h.u-tokyo.ac.jp
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Steroid hormone receptors

Nuclear receptors have common structural characteristics, *ie*, they contain N-terminal domain (A/B domain), DNA-binding domain (C domain), hinge domain (D domain), and the C-terminal domain or ligand-binding domain (E/F domain). The activation function 1 (AF-1) in the N-terminal domain has ligand-independent and constitutive transcriptional activity, while the ligand-binding domain contains ligand-dependent transcriptional activity (AF-2)[7, 8]. When steroid hormone receptors are not bound by their ligands, their transcriptional activity is inert due to their association with heat shock proteins. When binding to ligands, receptors will dissociate from the heat shock proteins and change their tertiary structure upon dimer formation, phosphorylation, and nuclear localization[9, 10]. Then, the receptors will bind to hormone response elements (HREs) existing in the transcriptional regulatory regions of their target genes[11]. The prototypic estrogen

response element (ERE) comprises a palindromic consensus sequence of AGGTCA motifs separated by a 3-base spacer. In the case of the androgen-response element (ARE), the palindromic consensus sequence of AGAACA motifs are separated by a 3-base spacer. Interestingly, AR, PR, GR, and MR recognize essentially the same consensus sequence. Ligand-bound nuclear receptors associate with transcriptional coactivators and, subsequently, recruit RNA polymerase and general transcription factors (Figure 1)[12].

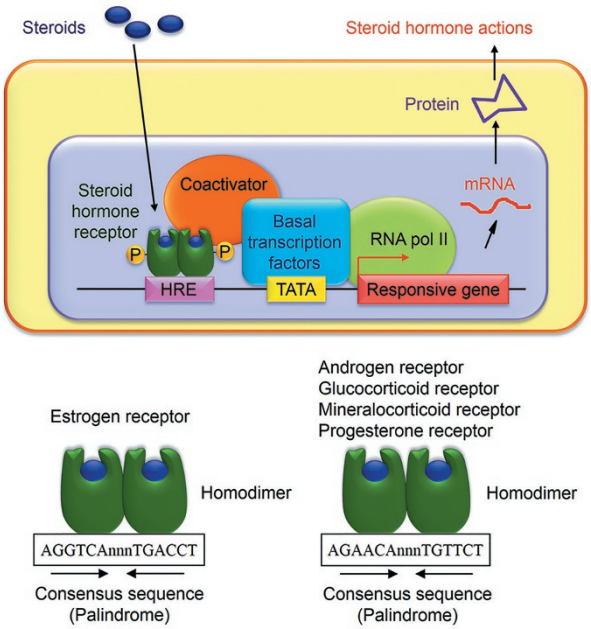

Figure 1. Transcriptional regulation mechanism of steroid hormone receptors. Binding of steroid hormone receptors to the hormone responsive element (HRE) is shown in the lower panel.

The coactivator comprises a protein complex that includes factors possessing histone acetylase or chromatin remodeling activity, which will loosen the chromatin structure and promote the recruitment of the transcription apparatus[13]. When the receptor is occupied with an antagonist or free from ligands, it is associated with a transcriptional corepressor that represses transcription[14]. Corepressors form a protein complex with histone deacetylases and exert their function to maintain a rigid histone structure. Both coactivators and corepressors are necessary for the effective control of transcription of the target gene. In addition, the nuclear receptor can regulate the transcription of target genes without binding to HREs, *ie*, the nuclear receptor is known to associate with other transcription factors such as Sp1 and AP-1 and then transactivate genes through the binding factors[15, 16]. Recently, it was shown that FOX family transcription factors influence ERα-regulated transcription by interaction with ERα protein, as exemplified by FOXA1[17]. Genome-wide studies aimed at identifying ERα and AR binding sites (ARBSs) have shown that FOXA1 plays a important role in regulating the networks of both of these nuclear receptors[18, 19]. FOXA1 is recognized as a pioneer transcription factor because chromatin binding by this protein can enable subsequent recruitment of ERs and ARs to the genome[19, 20].

Hormone-dependent cancer and endocrine therapy

Breast cancer is one of the representative hormone-dependent cancers. Approximately two-thirds of breast cancers express the ER, which plays a critical role in the growth of estrogen-dependent breast cancer cells[21]. The expression of ER relates to various histological characteristics of breast cancer. In general, low-grade breast cancers are usually ER-positive, whereas high-grade cancers are more commonly ER-negative[22]. ER-positive tumors usually have a better prognosis than ER-negative tumors. Recently, classification of breast cancer subtypes based on HER2 expression as well as ER and PR status was performed to determine the appropriate treatment and to predict the patient's prognosis[23]. In addition, it was shown that the expression patterns of several genes clinically correlate with the outcome of adjuvant therapy and the prognosis of breast cancer patients[24]. Thus, gene expression profiling will provide a new taxonomy of breast cancer. In the clinical setting, anti-estrogens and aromatase inhibitors are used in endocrine therapy to treat breast cancer. It was shown that tamoxifen binds to ER and represses its transcriptional activity in breast cancer cells. Aromatase inhibitors appear to decrease the estrogen level in breast cancer cells. Acquired resistance to tamoxifen and aromatase inhibitors, however, is a critical problem in the management of breast cancer, because the majority of patients with breast cancer are treated with these drugs[25]. Therefore, elucidation of the mechanisms underlying the estrogen-signaling network in breast cancer will provide useful information to predict the efficacy of endocrine therapy and the prognosis of cancer patients. In addition, functional analysis of estrogen-responsive genes in terms of growth, invasion, and metastasis in breast cancer cells will be useful for the development of new methods for the diagnosis and treatment of the disease.

Steroid hormone target genes and the transcription cascade

In the 1990s, identification of target genes of steroid hormone receptors was performed using DNA fragments binding to the receptors. In order to isolate estrogen-responsive genes, including EREs in their transcription regulatory regions, we have developed a technique called genomic binding-site cloning[26]. Using this method, several genomic sequences containing EREs were successfully isolated and, subsequently, novel estrogen-responsive genes were identified nearby functional EREs[27, 28]. Protein products of these genes include estrogen-responsive finger protein (Efp), cytochrome *c* oxidase subunit VIIa-related polypeptide (COX7RP), and estrogen receptor-binding fragment-associated antigen 9 (EBAG9). Abundant expression of Efp is often observed in breast tumors[29], and Efp is an estrogen-inducible gene in MCF7 breast cancer cells. Efp possesses a RING finger motif[29] and functions as

an ubiquitin ligase E3 for a negative cell cycle regulator 14-3-3σ protein, resulting in cell cycle progression via proteasome-dependent degradation of the 14-3-3σ protein[30]. Thus, Efp could function as an estrogen-responsive gene that stimulates the proliferation of breast cancer cells. Downregulation of Efp expression by small interfering RNA (siRNA) reagents will be useful for the management of breast cancer, as shown in mouse tumorigenesis models (Figure 2).

Figure 2. DNA-modified small interfering RNA (siRNA) against Efp effectively reduces the growth of a tumor derived from MCF-7 breast cancer cells injected in nude mice.

We have recently shown that FOXA1 and FOXP1 expression are estrogen-inducible and they promote the proliferation and/or migration of breast cancer cells by enhancing ERE-mediated transcription (Figure 3A). Genome-wide chromatin immunoprecipitation (ChIP) analysis on a DNA tiling array (ChIP-chip) showed the presence of estrogen receptor-binding sites (ERBSs) within the human FOXA1 and FOXP1 gene regions in MCF-7 cells[31]. These ERBSs involved in FOXA1 and FOXP1 loci were functional and estrogen-dependent in MCF-7 cells as confirmed by our conventional ChIP analysis. We further demonstrated that the immunoreactivity of both FOXA1 and FOXP1 (Figure 3B) positively correlated with distant disease-free survival for tamoxifen-treated patients of breast cancer[31]. These results suggest that pharmacological modulation of FOXA1 and FOXP1 activities would be clinically useful for preventing and/or treating breast cancer, and that evaluation of the immunoreactivity of both FOXA1 and FOXP1 could predict the therapeutic effect of tamoxifen in breast cancer patients.

In addition to the estrogen-responsive genes identified by our group, many other genes have been found to be estrogen-regulated, using techniques such as RT-PCR and Northern and Western blot analyses. However, among them, only a small number have been shown to possess functional EREs within the transcription regulatory region. In mammals, these genes include transcription factors, such as *JUN*[32], *FOS*[33], *PGR*[34], and *TP53*[35], intracellular signaling molecules, such as *HRAS*[36], *BCL2*[37], and *BRCA1*[38], enzymes, such as *CHAT*[39], *NQO1*[40], and *CKB*[41], secreted proteins, such as *LTF*[42], *SCGB1A1*[43], *OVGP1*[44], *C3*[45], and *AGT*[46], hormones, such as *LHB*[47], *OXT*[48], *PRL*[49], and *AVP*[50], membrane proteins, such as SNAT2[51] and VEGFA[52], the motogen TFF1[53], and the protease CTSD[54]. These genes are assumed to directly mediate various estrogen actions in normal tissues, as well as in cancer and other diseases. However, these genes appear insufficient to explain the estrogen action in breast cancer. Moreover, most of these genes contain an imperfect consensus palindromic ERE or the half site of ERE[55], suggesting that the sequences of EREs in natural promoters of estrogen-responsive genes are diverse. Therefore, genome-wide comprehensive analysis of ER binding sites and estrogen-responsive genes is required to fully elucidate the estrogen-signaling network in breast cancer.

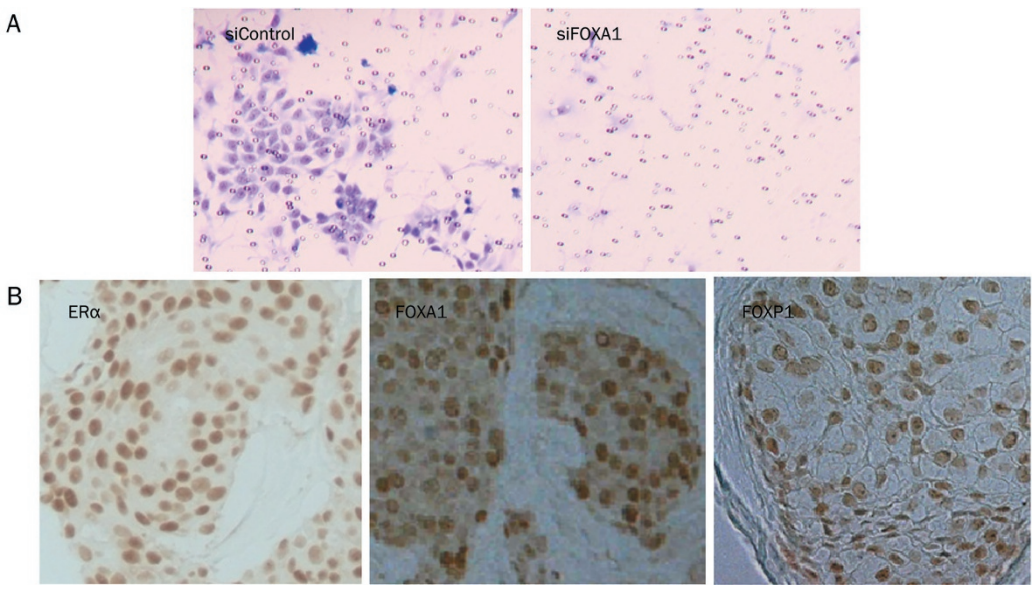

Figure 3. Forkhead transcription factors FOXA1 and FOXP1 contribute to the biology of breast cancer cells. (A) Migration of MCF-7 cells is repressed by small interfering RNA (siRNA)-mediated knockdown of FOXA1. (B) Estrogen receptor (ER) α, FOXA1, and FOXP1 immunoreactivities are predominantly observed in the nucleus.

Identification of estrogen-responsive genes using high-throughput sequencing techniques

Recent advances in high-throughput technologies have further revealed the genome-wide gene regulation mediated by ER. In addition to transcriptome analyses such as RNA-sequencing, CAGE sequencing[56], and microarray analysis, mapping of ER binding sites (ERBSs) is being performed by ChIP-chip or ChIP sequencing (ChIP-seq) analysis, which is a deep-sequencing technique for ChIP-derived DNA fragments[57]. Integrated studies of transcriptomes and transcription factor binding sites can provide useful information for ER-mediated gene regulation in a genome-wide manner[58]. In particular, the development of next-generation sequencing has allowed us to obtain DNA sequence information much more rapidly, and from across the genome. Gene expression microarray studies have been performed to identify the estrogen-responsive genes in breast cancer cells[59–62]. However, the effectiveness of expression microarray analysis is decreased by technical issues such as relatively high levels of noise and low sensitivity. In addition, expression microarrays typically do not cover the non-coding RNAs that have been implicated in gene regulation. RNA expression analysis using next-generation sequencing (RNA-seq) has resolved these issues, and enables a sensitive and unbiased determination of the estrogen regulation of genes[63, 64]. In this review, we discuss recent data concerning ERBS and estrogen-responsive genes in breast cancer which have primarily been revealed using high-throughput sequencing technology and ChIP-chip or ChIP-seq technology.

The first comprehensive genome-wide study of ERBSs was performed by Carroll and colleagues using ChIP-chip analysis. Their study identified 3665 ERBSs and 3629 RNA PolII binding sites, and demonstrated that binding of ER at multiple sites is a prerequisite for target gene regulation[59]. In addition, the binding motifs of transcription factors, including FOXA1, C/EBP, and Oct, have also shown to be positively correlated with ER[59]. Lin *et al* used ChIP-paired end diTag (ChIP-PET) technology and deep-sequencing for mapping ERBSs in the genome of MCF-7 cells[65]. In their report, 1234 high-confidence ERα-binding sites (ERBSs) were identified and several transcription factor binding motifs, including Sp1, AP-1, and FOXA1 were found to be enriched with the ERBSs. Welboren *et al* used ChIP-seq technology to explore ERBS and binding sites for RNA polymerase II in the MCF-7 genome in response to estradiol (E2) and antiestrogen, tamoxifen, or fulvestrant[66]. They identified the 10 205 E2-regulated ERBSs in MCF-7 cells and showed that tamoxifen and fulvestrant partially decrease the binding of ERα and RNA polymerase II around the E2-upregulated genes[66, 67]. To examine the role of FOXA1 in ERα binding, Hurtado *et al* performed ChIP-seq analysis of ER binding sites in MCF-7 cells in which FOXA1 was silenced with short interfering RNAs (siRNAs)[68]. Silencing of FOXA1 decreased the binding intensity at ERBSs that are in the vicinity of FOXA1 binding sites. In addition, silencing of FOXA1 affected the expression of estrogen-responsive genes. Hurtado *et al* also showed that FOXA1 is necessary for ERα binding in tamoxifen-regulated gene expression in breast cancer cells and that most ER binding sites overlap between estrogen- and tamoxifen-treated cells, implying that FOXA1 also plays a role in tamoxifen-mediated transcriptional regulation of ERα[68]. Moreover, they performed formaldehyde-assisted isolation of regulatory elements coupled with high-throughput sequencing (FAIRE-seq) to identify nucleosome-free and dense chromatin regions in MCF-7 cells and showed that ERα binds to nucleosome-dense regions in combination with FOXA1[68]. In addition to the finding that the binding of FOXA1 to ERE is essential for establishing stable recruitment of ER to EREs[68–72], these observations support the notion that the FOXA1 transcription factor functions as a pioneer factor for ER.

Comparative analysis of ChIP-seq data between ER and other transcription factors, such as ELF5, LRH-1, GATA-3, and TLE1, highlighted several key factors influencing ER-mediated gene transcription in breast cancer cells[73–75]. In addition to FOXA1, ChIP-seq analysis of FOXM1 revealed that FOXM1 binds at genomic sites simultaneously with ERα and regulates the transcriptional activity of ERα[76]. Genome-wide analysis of ER and retinoic acid receptor (RAR) binding sites in MCF7 cells also demonstrated that a large portion of ERBSs overlap with RAR receptors, suggesting a correlation between these two groups of nuclear receptors[77, 78]. ChIP-seq analysis of the coregulatory factors for ER, including the p160 family (SRC1, SRC2, and SRC3) and the histone acetyl transferases, p300 and CBP, were performed in MCF-7 cells and revealed a complex network of coregulatory factor binding. In particular, genes possessing preferential binding sites for SRC3 have predictive value for clinical outcome[79]. Moreover, in MCF7 cells, unliganded ERα binding to many sites in the chromatin is considered to be involved in maintenance of hormone-dependent breast cancer cells[80].

ChIP-seq technology has also been adapted to improve its application to clinical biopsy material from breast tumors[81]. Ross-Innes *et al* also explored ER binding sites in primary breast cancer using ChIP-seq analysis and identified differential ER-binding profiles that are correlated with good or poor clinical outcomes, suggesting that ChIP-seq analysis will be useful for providing predictive prognoses in breast cancer[82].

Estrogen-regulated gene transcription in MCF-7 cells has also been monitored using GRO-seq, which is a high-throughput sequencing method adapted from nuclear run-on assays. GRO-seq provides the position and orientation of mRNA transcripts engaged by RNA polymerases. Using GRO-seq analysis, Hah *et al* revealed that the expression of ~3000 protein-coding genes is regulated by estrogen in MCF-7 cells[83–85]. Combined GRO-seq and ChIP-seq analysis in MCF-7 cells has shown that half of the immediate early estrogen-responsive genes possesses ERBSs in the vicinity (10 kb) of the transcription start site, although other studies have shown that ERBSs are dispersed throughout the genome[59, 65, 66, 69, 83].

Data from our recent RNA-seq studies, combined with genome-wide ERBS data[59], were used to identify novel

estrogen-responsive genes associated with the biology of breast cancer cells. These studies were conducted in breast cancer MCF-7 cells before and after treatment with 17β-estradiol (E_2)[63], and >1000 genes were identified as estrogen-responsive genes whose expression was altered by at least 2-fold over basal levels at any time point after estrogen treatment. These genes include prototypic estrogen-responsive genes such as *GREB1* and progesterone receptor (*PGR*) as well as novel estrogen-responsive genes such as *RAB17* (Figure 4). Loss-of-function studies of these estrogen-responsive genes revealed that siRNAs targeting MYC, EIF3A, and CCND1 significantly attenuated the growth of MCF-7 cells, and siRNAs specific for RAB17, TPD52L1, MYC, EIF3A, and CCND1 significantly repressed migration of the cells. Similar to prototypic ERα target genes, such as *MYC* and *CCND1*, *RAB17*, *TPD52L1*, and *EIF3A* could be new candidate genes that promote the growth or migration of ERα-positive breast cancer cells. It is noteworthy that *RAB17* is a member of the Rab family of small GTPases and has been shown to be upregulated by knockdown of extracellular signal-regulated kinase (ERK) 2, which is involved in the growth, migration, and invasion of cancer cells[86]. Thus, *RAB17* appears to be correlated with a favorable prognosis for ER-positive breast cancer[87].

Conclusion

The responsive genes of nuclear receptors are vigorously analyzed in genome-wide studies elucidating the cascade of transcriptional regulation networks (Figure 5). Estrogen functions are exerted through the ER-mediated regulatory networks. In these networks, transcriptional regulatory factors common for these receptors are utilized. Furthermore, the networks are functionally modulated by signal transduction pathways in

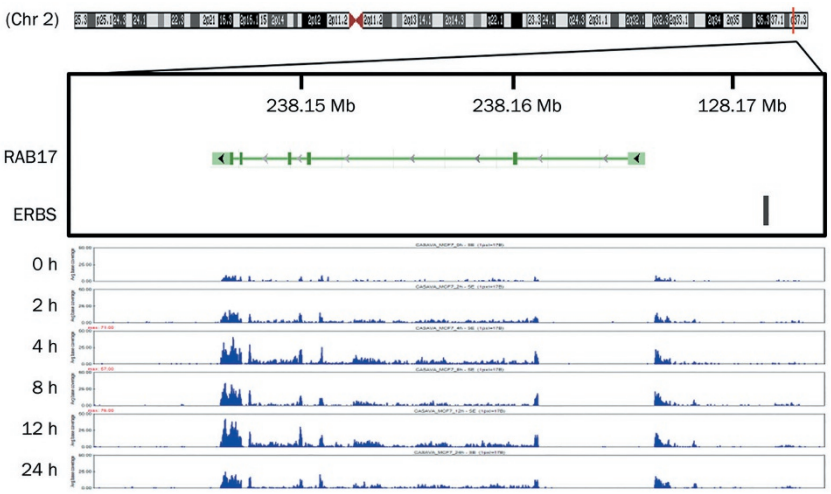

Figure 4. Estrogen-dependent upregulation of RAB17 in MCF-7 cells demonstrated by RNA-sequencing.

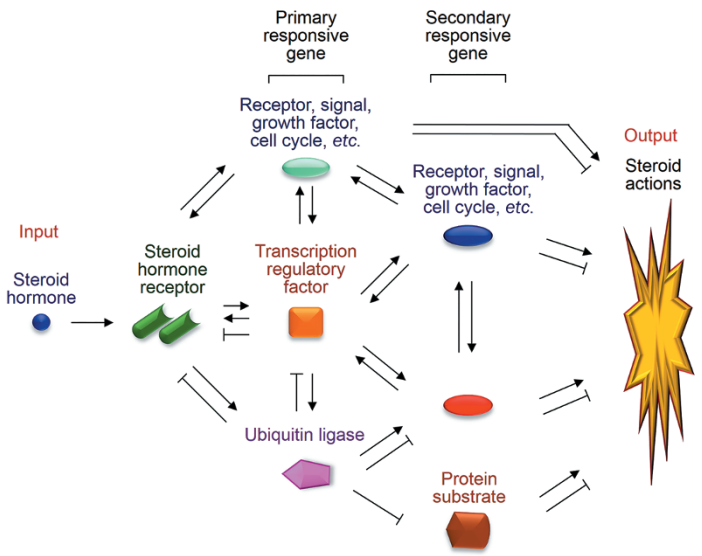

Figure 5. Transcriptional network of steroid hormone receptors and crosstalk with other signals.

a tissue/cell-specific manner. Genome-wide analysis using high-throughput techniques would be useful for clarifying the transcriptional network. In addition, functional and pathophysiological analyses of each gene involved in the network will reveal the role of the gene in cancer and diseases, which can be applied to the development of new diagnostic and therapeutic devices in clinical medicine.

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