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ABSTRACT

CTCF, Zn-finger protein, has been identified as multifunctional transcription factor to regulate gene expression through recruitment of other co-activators and binding to promoter regions of target genes. Furthermore, it has been proposed as an insulator protein that contributes to the establishment of functional three-dimensional chromatin structures. It could disrupt transcription through block the connection between enhancer and promoter. Previous studies revealed that various diseases including breast cancer were developed from aberrant expression of CTCF itself or their target genes. In this review, we will describe dysfunction of CTCF that induces tumorigenesis and summarize the functional roles of CTCF in breast cancer.

INTRODUCTION

CTCF is one of crucial chromatin organizer and is involved in transcriptional regulation by formation of enhancer-promoter looping. Genome-wide analysis such as Chromatin Immunoprecipitation Sequencing (ChIP-Seq), Chromosome conformation capture (3C) and Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET), serves dynamic roles of CTCF in gene regulation and genomic interaction. Global CTCF binding sites are normally conserved within tissues, however, specific CTCF bindings were identified and regulated by epigenetic factors in specific conditions. CTCF binding is strongly associated with DNA methylation status, and aberrant CTCF bindings depending on DNA methylation at IGF2/H19 locus lead to unexpected transcription to induce Beckwith-wiedemann syndrome (BWS) and Silver-russell syndrome (SRS). Since genes regulated by CTCF are related with proliferation and apoptosis, it has been studied the role of CTCF in various cancers including breast cancer. Breast cancer is heterogeneous disease associated with activity of hormone receptors, estrogen (ER), progesterone (PR), and HER2 (human epidermal growth factor receptor 2). Responsiveness of hormones is crucial pathological cause, and genes regulated by hormones are therapeutic targets for breast cancer. It has been investigated that genes regulated by ER are modulated by CTCF through binding to ER target genomic regions, constructing chromatin looping structures for enhancers and promoter interactions, limiting the ER influence when CTCF occupied with other proteins such as cohesion, ER, or BRG1 (1-4). Here, we will review recent studies of the CTCF function to understand mechanisms to regulate gene expression in breast cancer.

DIVERSE ROLES OF CTCF

CTCF is highly conserved protein which contains 11 zinc-fingers in eukaryotes, and recognizes specific binding sequences in genome. Genome-wide analysis indicated that half

of CTCF binding sites were observed in intergenic regions while the others were present in promoters and intragenic regions (3). This global analysis supports their multi-function as transcription activator/repressor, insulator, recombination modulator, and constructor to establish three-dimensional chromatin structure (5, 6).

It has been identified CTCF interacts with other co-factors such as homeodomain transcription factors (HOX-TFs) and glucocorticoid receptor (GR) at promoter or enhancer to regulate gene expression in primary mesenchymal limb bud cells and hepatic cells (7, 8). The association of CTCF and HOXA/D family transcription factors (TFs) bindings was observed in chicken genome (7). Among 9 HOX-TFs, 6 HOX-TFs were grouped by their coincident binding patterns with each other and their binding patterns were also comparable to CTCF binding sites in genome (7). GR and CTCF co-localization was confirmed when CTCF mediated chromatin structure was formed for communication between GR binding enhancers and promoter region of one of GR target gene, *ANGPL4*, for gene regulation (8). One of CTCF roles has been suggested as a boundary barrier to block transcriptional regulation in genome (9).

Insulator role of CTCF was revealed in specific developmental stages like B-cell and with specific co-occupied factors such as BRD2 (10, 11). CTCF could block the influence of rearrangement in *Tcrd* region in germline (12). And It was shown that B-cell developmental stages are affected by CTCF binding as an insulator. Reduced CTCF insulator activity induced to premature developmental process (10). CTCF insulator role is enforced when CTCF is co-occupied with BRD2, a member of bromodomain and extraterminal motif (BET) protein family. Loss of BRD2 showed the occasion of aberrant boundary architecture, even CTCF occupancy was not altered in *Slc25a37* locus. It could be explained both CTCF and BRD2 bindings work as an insulator, architectural boundary in genome to block enhancer regulation (11).

The function of CTCF associated with chromatin structure was shown by depleted CTCF. It led to aberrant chromatin folding structure in mouse embryonic stem cells (2). For maintaining stable genomic complex, CTCF could occupy on CTCF binding regions with connected proteins such as SMC1, SMC3, STAG1, STAG2, and RAD21 (13). Global analysis of protein binding sites was suggested that the shift binding patterns among CTCF, SMC3, and RAD1, it was related those protein-DNA binding motif sequences located in nearby each other (13). And the effect of CTCF was manifested that modulate chromatin looping structure for V(D)J recombination (12).

In a recent study, multi-functional CTCF was indicated at given situation in tandem. Eliminated CTCF binding was confirmed CTCF multi-function that CTCF is prominent factor for transcriptional regulation, distinct looping formation, and maintaining chromatin structure with cohesin in both inter-chromatin and intra-chromatin looping (2).

DISEASE-RELATED CTCF DYSFUNCTION

It has been identified that aberrant CTCF induces diseases or disorders including mental retardation, wiedemann syndrome, Silver-russell syndrome and various cancers (Table 1) (1, 14-19). Autosomal dominant mental retardation 21 (MRD21) is caused by frameshift mutation of the CTCF gene; c.375dupT and c.1186dupA and missense mutation at amino acid 567 from Arginine (R) to Tryptophan (W) leading to weaker binding affinity to DNA (14). These mutations showed distinct characteristics that involved short stature, microcephaly, mild facial dysmorphisms and various intellectual disabilities (16). Beckwith-wiedemann syndrome (BWS) and Silver-russell syndrome (SRS) are caused by abnormal CTCF bindings in imprinting control region (ICR) regulating *IGF2* and *H19* gene expression on chromosome 11p15.5 (15). It is closely connected with DNA methylation of ICR determining CTCF binding affinity. Paternal allele normally showed absent CTCF at methylated ICR that leads

to activation of *IGF2* whereas *IGF2* expression was inhibited by CTCF at unmethylated ICR on the maternal allele. Dysregulation of DNA methylation at ICR on maternal or paternal allele induces abnormal CTCF bindings leading to unsuspected transcription of *IGF2* and *H19*. Abnormal CTCF bindings depending on DNA methylation status in this locus was observed in testicular germ cell tumors (TGCT), colorectal cancer, bladder cancer, and ovarian cancer (19-23). In addition, it has been reported that SRS by dysfunction of CTCF on paternal allele induces developing specific malignant tumor such as hepatocellular carcinoma (24), Wilms' tumor (25), Testicular cancer (26) and craniopharyngioma (27).

And missense mutations of zinc-finger domain of *CTCF* gene were detected in various cancers including endometrial cancer, prostate cancer, Wilms' tumor, and breast cancer (1, 28-32). It has been investigated missense mutation R377C in endometrial cancer (28), H345R mutation in prostate cancer (29) and two missense mutations, R339W and R448Q, in Wilm's tumor (30). It has been also identified mutation in *CTCF* gene in breast cancer. K344E mutation (AAA→GAA), missense codon mutation in zinc-finger domain of CTCF gene, was observed in breast cancer (31, 32). We will mention more in next part.

CTCF/cohesion-binding sites (CBSs) mutations were investigated in various cancers including gastrointestinal cancer and skin cancer (33, 34). In gastrointestinal cancer, relatively A·T>C·G and A·T>G·C substitutions were preferentially detected at CTCF/cohesion-binding sites, and these mutations were related with late replication (19). Mutation caused by differential nucleotide excision repair (NER) across pyrimidine pairs was also identified at specific CBSs in skin cancer (34).

ABERRANT CTCF FUNCTION IN BREAST CANCER

As the association of CTCF and disease has been revealed in many studies, tumor cell growth

influenced by aberrant regulation of CTCF has been also observed in breast cancers significantly. Herein it is described about breast cancer causative dysregulation of CTCF cases. Not only mutations in zinc finger domain, but also abnormal regulation of *CTCF* gene expression and *CTCF* poly(ADP-Ribosyl)ation (PARlation) can influence to tumorigenesis in breast.

The studies have conducted to reveal the relation between mutations at CTCF gene region and breast cancer cell proliferation. CTCF has been recognized and studied as an important factor in pathogenic of breast cancer when disease-related heritable germline mutations were found in patients who suffered breast cancer. As mentioned above, missense codon mutation in *CTCF* zinc finger domain 3, K344E mutation was observed in breast cancer (31, 32). And it was also confirmed that K344E mutation could affect to unable CTCF binding to the promoter/insulator sites of cell proliferation related genes such as *MYC, PLK, PIM-1, p19ARF,* and *Igf2/H19* (1). It was suggested that *CTCF* mutations could aberrant CTCF formation and then it could influence to become DNA binding unable at known cell proliferation genes, and it could be breast carcinogenesis causative. In another study, two mutations were revealed by the association study of disease and DNA mutations. One is ^{240}G→A in 5' UTR and the other is S388S mutation, ^{1445}C→T, in exon 4 of *CTCF* gene were confirmed (35). DNA mutations at *CTCF* coding region could be the cause of breast cancer development by alteration of the expression level of *CTCF* (36). It was confirmed that somatic mutations including K344E, which are located at Zinc Finger DNA binding motifs of *CTCF* gene implicate breast cancers and it could be a reason of different protein formation and possibly to prevent typical function of CTCF. This study also showed CTCF function as a tumor suppressor gene by which enforced CTCF induced significantly inhibited carcinoma cell proliferation (36).

Furthermore, aberrantly expressed CTCF could be a major causative reason of

tumorigenesis in breast cancers. The effect of knockdown of CTCF was determined down regulated CTCF induces apoptotic cell death in breast cancer cells in MCF-7 (37). Cell survival was increased when sodium butyrate (NaB) was used to treat and CTCF was up-regulated in breast cancer cells. As the result, it was validated that CTCF expression level was related with cell survival against apoptotic cell death (37). *CTCF* PARlation was also matched with that result. Reduced *CTCF* PARlation also altered apoptosis and proliferation in breast cell lines and it could be a momentous feature in breast cancer cells during apoptosis (38). And translocation of CTCF was discovered from nucleoplasm to nucleoli whereby NaB treated MCF-7 (39). In a previous study (40), it was suggested that a 130 kDa CTCF could be used as a breast cancer biomarker, since both CTCF formations, a 130 kDa CTCF and a 180 kDa CTCF which is formed by PARlation, were detected in breast cancer tissues, while only a 180 kDa protein of CTCF was observed in normal tissues. A 130 kDa CTCF is negatively correlated with tumor size and tumor stage. When the transition formation of CTCF-130 level was increased, increased cell proliferation was also observed. By NaB treatment, it was verified that apoptosis was induced, 180 kDa CTCF was manifested, and cell proliferation was decreased in MCF-7 (40).

Few studies were shown that abnormally expressed CTCF which generated breast cancer cells, was regulated by DNA methylation in genome. Here is a study indicated that how DNA methylation and down-regulated CTCF affects to tumorigenesis gene regulation in breast tumors (28). In breast cancer, DNA methylation status of tumor suppressor genes and imprinting regulatory regions were aberrantly changed and it had an impact on decreased CTCF expression level. Especially, down regulated CTCF was positively correlated with dysregulated methylation pattern at CpGs nearby its gene of known tumorigenesis genes, such as *Trp53, Dnmt4A, RunX1* and *Ctcf* (28). This altered methylation status led to unavailable CTCF binding to CpG regions which have CTCF motif sequences nearby CTCF-

regulated genes. Thereby tumor growth is progressed by without CTCF modulation (28). And in another study, it was supposed that methylated status of *BRCA1* promoter caused *BRCA1* inactivation and that influenced to aberrant expression of *CTCF* and appeared novel cytoplasmic *CTCF* expression (41). However, there is an interesting study result which CTCF gene expression level is not altered by DNA methylation status of CTCF promoter region (42). To determine the relation between the expression level of CTCF and breast cancer types, primary breast cancer lesions were discriminated as several types, invasive lobular carcinoma (ILC), invasive ductal carcinoma (IDC), mucinous carcinoma, metaplastic carcinoma, and other invasive carcinomas and ductal carcinoma *in situ* (DCIS). Even methylation status at CTCF promoter sites had no discrepancy between breast cancer tissues and adjacent normal tissues, gene regulation of CTCF was significantly diminished in invasive breast cancer types. CTCF expression level was shown negative correlation with proliferation level especially in ILC compared to other carcinoma types (42).

It was verified not only methylation status could alter CTCF transcription levels. CTCF gene expression could be also differentially regulated by female sex hormone. Decreased CTCF expression level was proved when 17β-estradiol (E2) was exerted in MCF-7 breast cancer cell line. And decreasing level of CTCF was directly proportional to E2 exerted rate (43). It shows the possibility of indication that CTCF regulation could be affected by hormone regulation in breast cell lines.

DYNAMIC ROLES OF CTCF IN BREAST CANCER

Not even the distinct expression level of *CTCF*, interaction of CTCF and CTCF regulated genes is also possible to use as a marker of breast cancer. CTCF can affect to breast cancer development by regulating its target genes when it acts as a transcription factor, insulator, and/or regulator of chromatin structure by alone and/or with other factors (44).

CTCF binding to gene regulatory regions could be associated with the alteration of tumorigenesis genes expression level with co-regulatory factors and DNA methylation status (Figure 1A). A previous study showed when cell proliferation related genes such as *MYC*, *p19ARF*, and *Igf2* had no CTCF binding at promoter sites, the regulation of those genes could be altered(1). Consequentially, cell growth was also differently modulated to provide the malignant phenotype. It was certified that the correlation between CTCF depletion and apoptotic cell death in ZR-75-1 cells, but not in HeLa cells (45). The pro-apoptotic related gene, *Bax*, had unmethylated promoter in breast cancer cells, MCF-7 and ZR-75-1, and non-breast cancer cells, HeLa and 293T. Nevertheless, *Bax* expression level was increased in breast cancer cells which was deficient CTCF by siRNA. To testify the effect of *Bax* and *CTCF*, protein level of the cleavage of PARP-1, hallmark of apoptosis, was confirmed in Bax and CTCF double knockdown breast cancer cells, ZR-75-1 and MCF-7. As the result, apoptosis level was decreased in double knockdown cells compared to only CTCF knockdown cells. It supports that *Bax* overexpression leads to apoptotic cell death in decreased CTCF cells. *Bax* gene has two CTCF protein binding sites in its downstream of proximal promoter region which were enriched by CTCF in breast cancer cells. It is possible that Bax and CTCF act as negative regulators for each other (45). *HOXA10* promoter activity was also modulated by CTCF binding status (46). *HOXA* cluster are enriched by CTCF bindings in MCF-7. Of *HOXA* cluster, it was revealed that *HOXA10* had altered expression by CTCF expression inversely. *HOXA10* is sensitively regulated by CTCF binding at promoter regulatory region in which CTCF binding motif sequence is included. To verify whether CTCF enrichment status was negatively correlated with *HOXA10* expression levels, repressive and active histone markers, H3K27me3 and H3K4me3, were considered together. As the result, histone modification markers were coincided with *HOXA10* expression level. It was shown the increased H3K27me3 and decreased H3K4me3 modification mark in CTCF

overexpressed MCF-7 cells. And promoter had CTCF binding in MCF-7 and T-47D, but not in BT-474. It is the evidence that this sensitivity of CTCF binding is cell line specific depending on cell line specific *HOXA10* promoter regulatory region (46).

CTCF has been testified usually combined with demethylation CpG regions including its DNA binding motif sequences. (47, 48). In a previous study, it was suggested that CTCF binding modulated differentially presented DNA methylation status and histone modifications to regulate genes expression (49). Expression of X-linked inhibitor of the apoptosis (XIAP)–associated factor 1 (XAF) was controlled by CTCT binding with DNA methylation status and histone modification in proximal promoter site. It was confirmed that DNA methylation status was observed on CpG regions which was putative CTCF binding sites nearby proximal promoter region in which DNA methylation status could be changed by 5-aza-2'-deoxycytidine (5-A-DC), DNA demethylation modulator. The negative correlation was observed between *XAF1* expression levels and DNA methylation patterns. Enhanced CTCF associated with induced *XAF1* translational activity and demethylation status. Histone modifications, H3K27ac, H3K4me3, and H3K4me1 were observed to reflect altered transcriptional activities by CTCF binding and DNA methylation status (49). Methylation status could affect to CTCF bindings to regulate genes expression. Proximal upstream promoter site of retinoic acid receptor responder 1 (*RARRES1*), tumor suppressor gene, had methylated status where existed CTCF binding motifs in breast cancer cells such as MCF-7, SK-BR-3, but not in normal breast cancer cells, MCF-10A (50). It was examined the correlation between the gene expression level of *RARRES1* which was down-regulated in breast cancer cells with its hypermathylated proximal promoter region. *RARRES1* was reactivated by epigenetic drugs, DAC or TSA or both, in SUM159 but not in SK-BR-3. In SUM159, *RARRES1* reactivation led to increase cell death and prohibit cell invasion. *RARRES1* had CTCF binding to putative CTCF binding motif sequences with H3K4me2

occupancy at proximal promoter region. It was suggested that CTCF prevents DNA methylation and epigenetic silencing. And it was confirmed that deficient CTCF was affected to unable CTCF binding to *RARRES1* promoter region and decreasing *RARRES1* expression in MCF-10A cells. It could be delineated CTCF binding to *RARRES1* promoter was prominent for gene transcription (50). Delta-like ligand-4 (DLL4), a major gene for regulating notch signaling pathway, was also confirmed as a CTCF target gene (51). CTCF was highly expressed in MCF-7 compared to other cancers and CTCF was involved in regulating the gene expression level of DLL4 by binding to proximal promoter region. Even only CTCF is not enough to alter the expression levels of DLL4, it was shown that DLL4 was regulated by deficient condition of both CTCF and tumor suppressor gene, p53 (*TP53*) (51).

Recent studies have showed the relation between CTCF binding and not only the mRNAs, but also miRNAs. The expression levels of miRNAs were also modulated by CTCF binding, DNA methylation, and histone modification (52, 53). In a previous study, it was discovered that increased *miR-375* regulation interacted with active ER signaling (52). *miR-375* was expressed significantly up-regulated in ER positive breast cancer cells compared to ER negative breast cancer cells and normal breast cells. In breast cancer cells, the function of miR-375 was investigated through decreased cell proliferation when miR-375 was inhibited. By ER targeted siRNA, decreased signaling of ER led to reduced half of *miR-375* expression levels. It suggested that CTCF regulated *miR-375* gene expression by binding to CpG regions, especially first CpG island between two CpG regions. It was confirmed that ER positive cell lines, MCF-7 and T-47D, had distinguishable methylation status of two CpG islands, one was hypermethylated and the other was unmethylated compared to other cell lines. As enriched CTCF binding with H3K9me2 was presented at first CpG region in MCF-12A and MDA-MB-231 ER negative cell lines compared to MCF-7. Through gene expression profiling, Ras dexamethasone-induce 1 (*RASD1*) was indicated as a *miR-375* target gene had negative

correlation with ER regulation. Taken together, the loop of ER, *miR-375*, and *RASD1* associates with ER regulation (52). Another miRNA, *miR-125b1* was also known to affect to cell proliferation (54). In proximal promoter region of *miR-125b1* has also methylated CpG island and in approximately 90% of breast cancers, and the expression levels of *miR-125b1* was decreased in breast cancer cells (53). It could be illustrated DNA methylation status in CpG island are involved in tumorigenesis. This CpG island had CTCF binding in normal cells but not in breast cancers and H3K9me3 and H3K27me3, repressive histone modification marks, were localized in breast cancers. It could be explained methylation status, CTCF binding, and histone modification marks regulated *miR-125b1* expression. In breast cancers, CTCF binding was not able to locate on methylated CpG island and histone modification marks represented epigenetic status, therefore *miR-125b1* was not expressed (53).

CTCF could involve in regulating the expression level of CTCF modulated genes by acting as a transcriptional insulator to induce gene regulation to block the interaction of enhancers and promoters (9). Here is another investigation of CTCF bindings which were regulated independently of estrogen (55). It was found that estrogen regulated function was limited by CTCF binding sites as barriers. Among CTCF bindings, putative insulators were selected in *TFF* locus. In this locus, *TFF1* and *TFF2* were increased by E2, whereas other genes located outside of CTCF binding sites were not induced significantly by E2 (Figure 1B). And occupied CTCF bindings were not affected by E2 stimulation. It was shown that ERα was located at TFF1 promoter. This association was hampered when CTCF expression was reduced by siRNA. Even though E2 stimulation could not involve in CTCF binding alteration, DNA methylation status at barrier region could hinder CTCF occupancy. It was not observed any interaction the barrier CTCF bindings and any elements in outside of these barriers. This boundary CTCF binding interaction was not affect by ERα or HOA1. It could be explained those putative insulators construct chromatin structure as pioneer to restrict ER

regulation (55).

One of well-known CTCF function is modulating chromatin structure as a chromatin boundary factor for transcriptional activity regulation. CTCF constructs chromatin architecture with co-factors, such as cohesion complex including RAD21, SMC1 and SMC3 and BRG1 (4, 13, 56-58). Tang and colleagues revealed chromatin topological domain could be altered by CTCF binding which located to its specific DNA motif sequences (56). Cell line specific mutations in CTCF binding motifs shows different chromatin looping. Even BRG1 and CTCF co-localization is not abundant portion in their whole genome binding sites and their relation has not investigated particularly, it was observed BRG1 and CTCF interaction could preserve sturdy topologically associating domains (TAD) boundary (4). BRG1 is not particularly a ATPase subunit and one of major protein of SWI/SNF enzymes which are ATP-dependent chromatin remodeler. In recent studies, it was shown BRG1 could function to develop cancer cells in a different manner by hormone regulation in breast cancers (59, 60). In previous studies, it was shown BRG1 overexpression had positive correlation with poor prognosis in breast cancer cells (60, 61). Deficient BRG1 was revealed it affected to inhibit cell proliferation through cell cycle arrest, especially G1 phase, caused by decreased cyclin D1 and cyclin E, and increased p27 expression (60). Recently, BRG1 was investigated and the role was confirmed that BRG1 regulation was essential to preserve the topologically associating domains (TAD) with CTCF binding in normal breast cells, MCF-10A and MEF (4). Loss of BRG1 was used to confirm that BRG1 was a causative factor in week formation of TAD boundary and reduced nucleosome around CTCF bindings. The strength of BRG1 and CTCF interaction was enhanced when they located within 1kb from each other. It could be explained dysregulated BRG1 expression may associated with discomposed chromatin structure, especially regulatory regions for cell proliferation in breast cancer cells. Consequentially, it could be suggested that related genes could be expressed differentially and

then, it could result in altering cell proliferation rate (4). Synthetically, substantial chromatin structure could be maintained when CTCF and significant co-factors are located together. Even it is remained to be investigated that the direct effect of aberrant chromatin structure and attenuated chromatin looping domain by CTCF binding in breast cancer. It could affect to exceptional transcriptional regulation and then it could alter significant gene expression led to tumorigenesis.

Multifunctional CTCF is also associated with alternative splicing variations. It was proved the function of CTCF binding to promote RNA Polymerase II (Pol II) pausing and regulate alternative splicing at exon 5 of CD45 gene in murine splenocytes (6, 62). In a previous study, the relation between CTCF binding and alternative promoter (AP) activity was also suggested in estrogen regulated breast cancer cells, MCF-7 (63). Among estrogen-regulated genes, AP regulation was observed, especially in differentially expressed and regulated genes in MCF-7. In nearby most of these genes, it was appeared that specific ER regulate regions and CTCF bindings. Thereby, it was causative in AP regulation. For example, *NET1* gene had two different AP formation, one is transcribed from exon 1 (AP-1) and the other is transcribed from exon 4 (AP-2) regulates *NET1* long-form and short-form transcripts (Figure 1C). AP regulation of *NET1* was affected by estrogen stimulation with ER co-regulator factors, DDX5 and DDX17, and CTCF binding. It was observed that specific ER binding site at 8kb downstream and CTCF binding between exon 3 and exon 4. AP-1 and AP-2 activity could be separated by CTCF binding. AP-1 was highly expressed in ER stimulation which regulated without exon 4 expression, whereas AP-2 expression levels were decreased. RNA Polymerase II (Pol II) levels and mRNA levels were correlated with the AP regulation. Obviously, when AP-1 was increased by ER stimulation in which it was also observed advanced Pol II levels, while Pol II activity was decreased in AP-2. Indeed, AP regulation could be possible to produce distinct protein formations which are involved in different

function in cells. And cell growth and prognosis of patients were influenced by long-form and short-form expression levels of *NET1* transcripts. As the result, it was confirmed that AP activity could led to aberrant genes regulation and be regulated by CTCF modulation with estrogen stimulation effect and ER related DDX5 and DDX17 co-regulator influence (63).

A previous study (64) showed CTCF has correlation with CTCF and ER in breast cancer cell lines. Even in breast cancers, it was observed that CTCF and ER binding patterns are different depending on cell lines, breast cancer subtypes. Cell line specific CTCF binding is co-occupied with cell line specific ER binding in breast cancer cell line, MCF-7 (64). Most of ER and CTCF co-binding sites were localized within 20kb from estrogen-regulated genes. Thereby, it could be explained estrogen-mediated gene expression level could be regulated by ER and CTCF. Fiorito and colleagues studied CTCF binding enrichment is modulated by ER stimulation to impede the expanded loops of ER regulated enhancers and promoters of genes in MCF-7 (3). It was confirmed the alteration of chromatin looping depending on estrogen injection time. And CTCF binding sites were increased at intergenic regions as estrogen injection time goes by. When estrogen-induced transcripts were highly expressed at specific time points, CTCF binding density was also extremely increased. ER has prominent function for intra-chromosomal interaction to regulate ER target genes. ER-ER chromatin looping was investigated using Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET), it showed CTCF binding were co-localized with ER binding for ER-ER loops. CTCF occupancy was observed in more than half of ER looping regions. By Chromosome conformation capture (3C)-PCR, 24% increased ER bindings was confirmed to induce in CTCF depleted cells. For example, *P2RY2* involved in many functions including cell proliferation and apoptosis was one of induced genes by estrogen stimulation, whereas not expressed in non-stimulated status (Figure 1D). Estrogen injection was influence to appear three ER bindings and specific ER-ER looping was increased in CTCF depletion condition

nearby *P2RY2*. Among the proteins which interacted with CTCF protein, significantly increased interaction of nuclear lamina related proteins and CTCF was observed. Interestingly, Lamin B binding was also confirmed at three ER binding sites nearby *P2RY2*. It could be explained that CTCF repressive ER-ER looping function was related with nuclear lamina to modulate chromatin structure. It could be described that CTCF hider the ER-ER looping structure to modulate ER target gene regulation. The expression levels of cell growth related genes were significantly changed depending on CTCF and estrogen stimulation. Even ER binding sites were existed nearby target genes, located CTCF at enhancer regions which transcripts eRNA, can inhibit ER looping formation. As the result, transcription and cell growth could be changed. (3).

POTENTIAL THERAPEUTIC TARGET FOR BREAST CANCER

In breast cancer cells, CTCF regulation has been revealed that it is related with dysregulated genes expression which are especially clustered as apoptosis associated functional genes and the effect of sodium butyrate (NaB) to CTCF regulation was confirmed that it was correlated with CTCF overexpression and induced cell transfection and apoptosis gene regulation (37). And NaB treatment led to increased CTCF-130 and cell proliferation and apoptosis reduction in MCF-7 by CTCF translocation (40). Above mentioned, CTCF bindings are sensitive to DNA methylation status at target binding sites, indeed CTCF was not able to occupy to methylated regions (45, 49, 50, 52, 53, 55). Therefore, DNA methylation related drugs such as 5-A-DC, could be considered as putative target treatment in breast cancers. In a previous study, Sulforaphane (SFN) was manifested that has effect to down-regulation of human telomerase reverse transcriptase (*hTERT)* gene which expressed in 90% of cancer cells, including breast cancer, but not in normal cells, through modulating methylation status of CpG site at its CTCF binding region (65). It showed SFN inhibited histone deacethylase

(HDAC) and induced CpGs demethylation, sequentially SFN-mediated epigenetic regulation affected to differentially adjusted transcription factor binding. As the result, *hTERT* was down-regulated in MCF-7 and MDA-MB-231 cells, but not significantly changed in MDA-10A (65).

However, even CTCF is known as prominent factor to prevent or induce tumorigenesis by oncogenes or tumor suppressor genes regulation, the study of CTCF target treatment is not prevalence. Otherwise, Brother of the Regulator of Imprinted Sites (BORIS) has studied as more common target of cancer treatment. BORIS, also called CTCF-like protein (CTCFL), shared conserved 11 zinc finger domains with CTCF, which are related with CTCF binding motif sequences (66). *BORIS* gene regulation is depending on *CTCF* regulation. The expression levels of genes are in complementary relation (66). As mentioned above, up-regulated CTCF helps to protect from apoptotic cell death (37, 45). Reversely, induced *BORIS* expression levels were coincided with tumor progression and silenced BORIS expression was correlated with increased apoptosis (44). Even BORIS is not observed in most cancers, aberrant expression of BORIS was observed in breast cancer cells (67). It indicates not only CTCF regulation, but also BORIS expression was also crucial to modulate cell viability in cancer cells (36). It was suggested BORIS targeted siRNA, using zinc finger domain targeting, could affect to apoptosis and the result was shown that reduced BORIS was implicated in induced cell death (68). It implies specific modified CTCF regulation is also used possibly as a target to modulate cell apoptosis in breast cancers.

CONCLUSION

CTCF is a dynamic functional protein that operates transcriptional regulation through working as transcription factors, insulators and chromatin looping structure. Abnormal CTCF's function caused by mutations of CTCF gene itself or their binding sites leads to

diverse disease including cancers. In breast cancer, it has been identified missense codon mutations or decreased poly(ADP-Ribosyl)ation of CTCF, and aberrant CTCF bindings were observed to lead to unexpected gene regulation by blocking enhancer activity. Even though previous studies revealed dysfunction of CTCF results in breast cancer, it is still remained to identify how CTCF regulates chromatin looping structure in specific locus that genes related to tumorigenesis are located. Disrupt CTCF expression by siRNA could not testify to CTCF regulation in formation of chromatin looping at specific locus. In addition, it could be also continued to reveal potential role of CTCF as therapeutic target for breast cancer since numerous genes regulated by CTCF are associated with proliferation and apoptosis in breast cancer cells.

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CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

FIGURE LEGENDS

Figure 1. The multiple functions of CTCF in breast cancer cells.

(A) CTCF as transcription regulator regulates target gene expression through binding with co-factors such as ER and TP53 at unmethylated CpG regions.

(B) CTCF as insulator prevents enhancer activity regulated by ER to neighboring genes, *TFF3* and *TMPRSS3*, at *TFF* locus.

(C) CTCF is involved in inverse expression of genes which have alternative promoters. After ER treatment, long-form transcript of *NET1* was highly expressed whereas short-form transcript separated by CTCF was decreased.

(D) Decreased CTCF after ER treatment induces chromatin looping to connect distal and proximal ERs at *P2RY2* locus.

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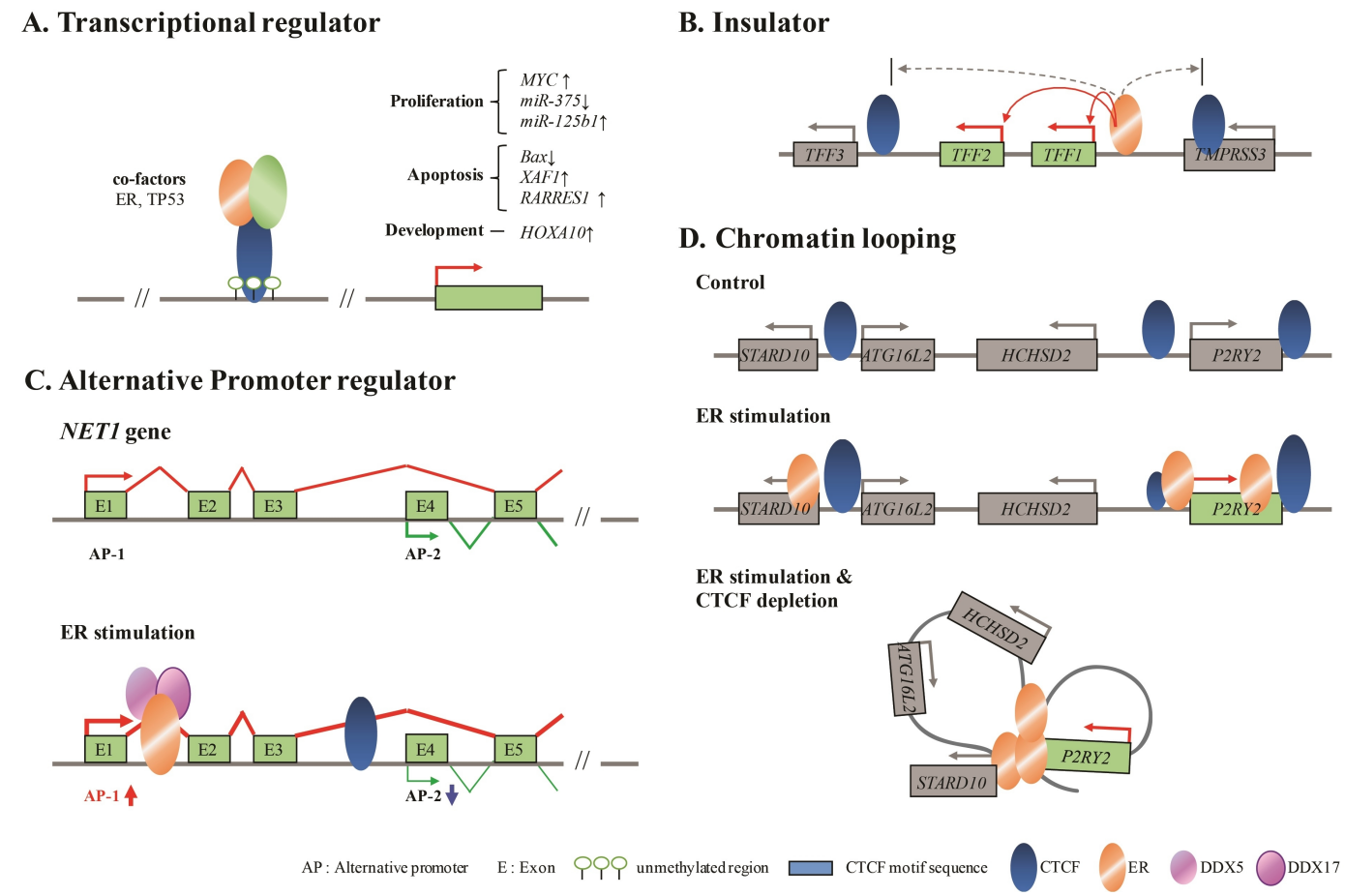

A. Transcriptional regulator
Proliferation
MYC↑
miR-375↓
miR-125b1↑
Apoptosis
Bax↓
XAF1↑
RARRES1↑
Development
HOXA10↑
co-factors
ER, TP53
B. Insulator
TFF3
TFF2
TFF1
TMPRSS3
C. Alternative Promoter regulator
NET1 gene
E1
E2
E3
E4
E5
AP-1
AP-2
ER stimulation
D. Chromatin looping
Control
STARD10
ATG16L2
HCHSD2
P2RY2
ER stimulation
ER stimulation & CTCF depletion
AP : Alternative promoter
E : Exon
unmethylated region
CTCF motif sequence
CTCF
ER
DDX5
DDX17

Fig. 1