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CTCF negatively regulates HOXA10 expression in breast cancer cells

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ABSTRACT

HOX genes not only play important roles in defining body patterning during embryonic development, but also control numerous cellular events in adult cells. Deregulated *HOX* gene expression in different cancers including breast cancer is now increasingly being reported. Given that human *HOXA* cluster is marked with several CTCF binding sites, we investigated whether the presence of CTCF is associated directly with expression of *HOXA* genes in breast cancer cells. Several *HOX* genes, such as *HOXA4*, *HOXA5* and *HOXA10*, were deregulated by CTCF overexpression and knockdown in MCF-7 cells. Among these genes, HOXA10 is an emerging tumor suppressor for its role in activation of p53 and in countering tumorigenesis in breast cancer. Here we provided evidences that CTCF functions as a negative regulator of *HOXA10* in breast cancer cells. The putative promoter region of *HOXA10* lies between 5.3 and 6.1 kb upstream of its start codon and its promoter activity was negatively regulated by CTCF. Together with in-silico analysis and in vitro mutation assay we identified a 20 bp CTCF binding motif flanking with core promoter element of *HOXA10*. *HOXA10* promoter region was kept inactivated by maintaining H3K27me3 inactivation marks in the presence of CTCF. Epigenetic silencing of *HOXA10* by CTCF in breast cancer cells may contribute towards tumorigenesis by decreasing apoptosis and promoting metastasis.

1. Introduction

HOX constitute a family of 39 transcription factors organized into four paralogous groups that play key roles in embryonic development. In adults, the expression pattern of HOX genes were conserved and maintained in a tissue specific manner and gave unique morphological identities to the cells [1–3]. HOX proteins regulate several key cellular functions such as cell adhesion, migration, and cell cycle to maintain cellular homeostasis [4]. Several HOX genes are found to be deregulated in different cancers and some of them have already established their roles in breast cancer tumorigenesis and progression [5,6].

Many HOX genes including an emerging tumor suppressor HOXA10 are silenced in patients with hereditary breast cancer [7]. It induces cycle arrest by transcriptional activation of p21 in U937 cells [8]. HOXA10 is also known to regulate p53 in breast cancer cells [6] and its down-regulation increases cell migration and metastasis [3]. These studies strengthen the role of HOXA10 as a tumor suppressor but the molecular mechanism of how HOXA10 is regulated in breast cancer cells is still elusive.

CCCTC binding factor (CTCF) is a highly conserved and ubiquitous transcription factor known to be involved in various unrelated cellular functions [9,10]. Its main functions are transcriptional activation, repression and enhancer blocking, depending on the genetic locus involved [11]. Originally discovered as a transcriptional repressor of chicken c-myc gene [12], CTCF is also known to inhibit transcription of several genes, such as *hTERT* and *Bax*, by binding near to their promoter regions [13,14]. Human *HOX* gene clusters contain several CTCF binding sites and deletion of CTCF binding site(s) within *HOXA* cluster resulted in expansion of active chromatin into the repressive domains [15]. CTCF functions as a controller of *HOXA* cluster silencing and maintains discrete functional domains of *HOXA* via long range chromatin interaction in human cells [16,17]. Apart from its role in establishing higher order chromatin structure, the presence of CTCF near the regulatory regions highlights its role as a transcriptional regulator in candidate genes.

In this study we investigated the role of CTCF as a transcription factor in regulation of *HOXA* gene cluster. Our work supports the idea that *HOXA10* expression is modulated by CTCF in breast cancer cells. Considering its role as a potential tumor suppressor, an insight of the mechanism of *HOXA10* regulation can be manipulated

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to activate cellular defense mechanism to induce cell cycle arrest and apoptosis in breast cancer.

2. Materials and methods

2.1. Cell lines and cell culture

MCF-7, BT-474 and T-47D cells were kindly provided by Drs. Yong Nyun Kim and Kyung Tae Kim (National Cancer Center, Korea). All the breast cancer cell lines were maintained as described in Ref. [18].

2.2. RNA isolation and RT-PCR

Total RNA from the cultured cells was isolated using Trizol reagent (Invitrogen Carlsbad, CA, USA) according to the manufacturer's instruction. ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA) was used for reverse transcription. PCR was performed in replicates using G Taq polymerase (Cosmogenetech, Seoul, Korea). PCR primers for *HOX* genes were previously reported [18]. Primers for other genes were listed in Supplementary Table T3. For quantification, ImageJ software was used.

2.3. Overexpression and knockdown of CTCF

Full length CTCF cDNA was cloned into pCDNA3 vector to obtain pCTCF recombinant plasmid. Transfection was done using Lipofectamin 2000™ (Invitrogen). Overexpression of CTCF was confirmed by RT-PCR and western blot analysis using CTCF antibody (Cell signaling, #3418). For CTCF knockdown, shRNA oligos targeting two different positions on CTCF mRNA (shCTCF-1; 5′-gcggaaagtgaacccatgata-3′, shCTCF-2; 5′- cctcctgaggaatcaccttaa-3′) were designed, synthesized by Cosmo GENETECH and cloned into pLKO.1 vector. Packaging plasmid (pCMV-dR8.91) and envelop plasmid (pVSV-G) were co-transfected with control (pLKO.1) or non-specific (NS) shRNA containing vector (pLKO.1-NS) or shCTCF containing vector (pLKO.1-shCTCF1 or pLKO.1-shCTCF2) in HEK-293T cells. Lentiviruses from the media were transduced into the breast cancer cells. Selection was done using 1.5 μg/ml puromycine for 48 h and CTCF knockdown was confirmed by RT-PCR.

2.4. Dual luciferase assay

Different genomic DNA fragments (Supplementary Table T1) from *HOXA10* locus were amplified using pfu polymerase (Solgent) and cloned into pGL3-basic vector (Promega) using *Kpn*I and *Hin*dIII sites. HEK-293T and MCF-7 cells were co-transfected with Renilla luciferase vector (pRL Renilla luciferase control) and either of control pGL3-basic vector or individual luciferase genomic construct using Attractene (QIAGEN). Promoter activity of individual construct was measured using Dual luciferase assay system (Promega) after 72 h of transfection and normalized using Renilla luciferase signal. The effect of CTCF on the promoter activity for pGL3-A10-2 vector was analyzed in MCF-7 cells with same methods.

2.5. In vitro mutation assay (IMA)

Wild type pGL3-A10-2 plasmid was mutated using PCR based introduction of mutation(s) to generate pGL3-A10-2-m1, pGL3-A10-2-m2 and pGL3-A10-2-m3 plasmids. Primers with mutated sequences F2 and R2 were used to amplify DNA fragments with their corresponding F1 and R1 primers as shown in Supplementary Fig. S5A and Supplementary Table T2. Resulting sub-fragment DNA were purified and used as a template for assembly PCR with F1 and R1 primers to generate mutated A10-2 fragment (Supplementary Fig. S5B). A10-2-mutated fragments are later cloned back into pGL3-basic vector for Dual luciferase assay (Supplementary Fig. S5C).

2.6. Chromatin immunoprecipitation (ChIP) analysis

Chromatin for ChIP experiment was prepared from MCF-7, BT-474 and T-47D cells by fixing the cells in 37% formaldehyde for 15 min, followed by quenching with 2.5 M Glycine for 10 min. Sonication was done using Sonics Vibra Cell™ to get 500–1000 bp DNA fragments (Supplementary Fig. S4A) with condition 1 (Time: 7 min, Pulse: 10 s Interval 10 s). In case of modified ChIP experiment (Supplementary Fig. S4B) DNA fragment up to 250 bp were generated using Condition 2 and 3 (Time: 9 min and 11 min, respectively, for condition 2 and 3 with same pulse and interval as condition 1). ChIP was performed using antibodies against CTCF, H3K4me3 (abcam, #1012), H3K27me3 (abcam, #6002) and IgG (Santa Cruz Biotechnology, sc 2027). Immunoprecipitated DNA was purified and used for PCR. Primers corresponding to CBS1 and CBS2 sites (Fig. 3A) are given in Supplementary Table T3. (For interpretation of the references to clour in this figure legend, the reader is referred to the web version of this article).

2.7. Immunocytochemistry (ICC)

MCF-7, BT-474 and T-47D were prepared using abcam ICC protocol. HOXA10 primary antibody (Santa Cruz, sc-17158) was used and detected by secondary antibody (Abcam, ab150129). Cells were counter stain with DAPI (invitrogen™) and analyzed using Zeiss LSM700 Confocal microscope.

2.8. Statistical analysis

For all the experiments, minimum three independent biological replicates were analyzed for quantitation. Data are presented as mean ± standard error. Student's t-test was used to calculate *p*-value. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. CTCF regulates expression of HOXA cluster in MCF-7

CTCF ChIP-Seq data in MCF-7 cells [19] adopted from Encode genome browser showed that several CTCF binding sites were present in *HOXA* cluster (Fig. 1A). This indicates a possible role of CTCF in regulation and maintenance of *HOXA* cluster expression pattern in MCF-7. In order to find a strong candidate gene regulated by CTCF, we first performed gain and loss of function studies and examined the effect of CTCF on the expression of *HOXA* cluster genes (Fig. 1). The overexpression of CTCF was validated by western blotting (Fig. 1B). *HOXA5* was upregulated while *HOXA4* and *HOXA10* were downregulated in CTCF over-expressing MCF-7 cells, expression of other genes were not changed (Fig. 1C *left* &D). When CTCF was knocked down, *HOXA10* was upregulated (Fig. 1C *right* & E). ICC data proved that HOXA10 nuclear and cytoplasmic expression was also increased after CTCF knockdown in MCF-7 cells (Supplementary Fig. S1). HOXA10, in particular, has been proposed as a tumor suppressor gene but not well studied for its regulation mechanism; we therefore selected *HOXA10* as a candidate gene to explore further for its regulation by CTCF.

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