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The role of Sp1 and EZH2 in the regulation of *LMX1A* in cervical cancer cells

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ABSTRACT

We have reported previously that LIM homeobox transcription factor 1α (*LMX1A*) is hypermethylated and functions as a metastasis suppressor in cervical cancer cells. However, the regulation of *LMX1A* in carcinogenesis has not been reported. We aim to clarify whether specificity protein 1 (Sp1) and enhancer of zeste homolog 2 (EZH2) are involved in the regulation of *LMX1A* in cervical cancer. First we characterized the *LMX1A* promoter and used overexpression, knockdown, and reporter assays to show that Sp1 increased *LMX1A* promoter activity. Next, we used site-directed mutagenesis and electrophoresis mobility shift assays (EMSAs) to demonstrate that Sp1-binding sites were important for Sp1-mediated activation of the *LMX1A* promoter. Chromatin immunoprecipitation data demonstrated that Sp1 could bind directly to the *LMX1A* promoter and activate endogenous *LMX1A* expression in cells pretreated with 5-aza-2′-deoxycytidine (5-aza-dC). Knockdown of EZH2 decreased H3K27me3 histone modification but was insufficient to restore *LMX1A* expression. To explore the effect of EZH2 on the endogenous *LMX1A* promoter, we treated EZH2-knockdown cells with 5-aza-dC and trichostatin A (TSA) and then depleted the cells of drugs for 3 days. H3K14ac was enriched at the *LMX1A* promoter in EZH2-knockdown cells and *LMX1A* mRNA was still expressed. Taken together, these data imply that Sp1 may activate *LMX1A* expression upon oncogenic stress during cervical cancer development. Moreover, suppression of EZH2 may delay resilencing of *LMX1A* after the removal of 5-aza-dC and TSA.

1. Introduction

High-risk specific types of human papillomavirus (HPV) are associated with cervical cancer progression in humans [1]. The transforming potential of oncogenic HPVs is closely related to the viral E6 and E7 oncoproteins, which inactivate p53 and retinoblastoma proteins (Rb) [2]. Extensive studies have shown that expression of the viral oncogene is essential but not sufficient for cancer development. Many reports have demonstrated that both genetic changes and epigenetic modifications play important roles in complex signaling pathways in cervical carcinogenesis [3–5].

Loss of function and gain of function through genetic alterations, such as mutations, deletions, copy-number aberrations, and chromosomal rearrangements, are associated with cancer progression [6]. In addition, epigenetic mechanisms that modify chromatin structure, including DNA methylation, covalent histone modifications, incorporation of histone variants, nucleosome remodeling, and noncoding RNAs, are also involved in carcinogenesis. Covalent histone modifications are posttranslational modifications of histone proteins mediated by enzymes that can modify covalent attachments at specific residues. In many cancers, dysregulation of epigenetic modifications, such as promoter hypermethylation and chromatin condensation, can lead to the activation of oncogenes and silencing of tumor suppressors [7–9].

Enhancer of zeste homolog 2 (EZH2), one of the histone methyltransferases (HMTs), encodes the catalytic subunit of the polycomb repressive complex 2 (PRC2). EZH2 contains the signature SET domain, which methylates lysine 27 of histone H3 (H3-K27), as a repressive marker. EZH2 and H3-K27 methylation are implicated in mammalian X chromosome inactivation and germline silencing [10]. Moreover, high EZH2 expression correlates strongly with cellular transformation, tumor progression, and poor prognosis [11–15]. Silencing EZH2 expression leads to reexpression of tumor suppressor genes by decreasing the content of trimethylated histone 3K27 (H3K27me3) in many cancer cell lines [16–18]. Some studies have also shown that EZH2 can physically interact with the DNA methyltransferases (DNMTs), facilitate their binding to EZH2-target promoters, and affect the DNA methylation level of the target genes of EZH2 [19,20]. Aberrations in DNA methylation and histone modification are also observed in the promoter region of genes and may inactivate tumor suppressor

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genes or activate oncogenes in cervical cancer [21–24]. HPV16 E6 could enhance DNMT1 expression through repression of p53 [25]. Furthermore, EZH2 expression is activated by HPV16 E7 at the transcriptional level via E7-mediated release of E2F from pocket proteins [26]. EZH2 is significantly upregulated in cervical cancer tissues, and knockdown of EZH2 can inhibit colony formation and invasion ability [26,27]. Moreover, *CDH1* (E-cadherin), regulated by EZH2, has been linked to cancer invasion and metastasis [28,29]. Taken together, we suggest that EZH2 plays important roles in tumor progression in cervical cancer.

In our previous study, we have characterized a gene, LIM homeobox transcription factor 1α (*LMX1A*), which is hypermethylated in squamous cell carcinomas (SCC) and functions as a metastasis suppressor in cervical cancer [21,30]. The expression of *LMX1A* protein is low in normal parts of the cervical epithelium but is high in cervical intraepithelial neoplasia (CIN) and carcinoma in situ (CIS), and is reduced markedly in invasive and metastatic cancer cells [30]. The specificity protein 1 (Sp1) protein expression pattern is similar to that of *LMX1A* in cervical cancer [31]. Sp1 belongs to the specificity protein/Krüppel-like factor (Sp/KLF) transcription factor family, which interacts with the GC-rich (5′-GGGGCGGGG-3′) motif in promoters. The DNA binding domain of Sp1 comprises three adjacent zinc fingers of the classical Cys2–His2 type, is expressed ubiquitously in many tissues, and is involved in multiple signal pathways [32,33]. Overexpression of Sp1 can have positive or negative effects on cell growth [34]. Sp1 has been implicated in invasion, metastasis, proliferation, and oncogenesis, and has antitumor activities [34–37]. These findings lead us to postulate that there might be a link between *LMX1A* and Sp1 in the development of SCC.

LMX1A is one of the LIM homeobox-containing genes and plays an important role during development. More recently, *LMX1A* has been reported as a metastasis suppressor or tumor suppressor. However, the regulation of *LMX1A* in carcinogenesis remains unclear. Therefore, we want to clarify the mechanisms responsible for the regulation of *LMX1A* in cervical cancer. Because the role of Sp1 in cellular transformation is contradictory, and several predicted Sp1 sites are located in the *LMX1A* promoter region, we propose that Sp1 can modulate *LMX1A* expression in cervical cancer. Moreover, the EZH2 expression pattern is highly associated with tumor cell invasion in cervical cancer. Our preliminary data showed that overexpression of EZH2 could repress *LMX1A* expression in cancer cells (Huh7). This suggests that EZH2 might be also involved in the regulation of *LMX1A*. In this study, we found that Sp1 bound directly to the *LMX1A* promoter and activated endogenous *LMX1A* expression when the methylation level of promoter was low. We also found that *EZH2* could regulate *LMX1A* expression through modification of chromatin status.

2. Materials and methods

2.1. Cell culture

HeLa, HeLa^{3rd}, SiHa, CaSki, C33A human cervical cancer cell lines, and Huh7 human hepatocellular carcinoma (HCC) cell line were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 supplemented with 10% (w/v) fetal bovine serum (Invitrogen, Carlsbad, CA, USA), penicillin at 100 U/ml, streptomycin at 100 μg/ml and L-glutamine at 2 mmol/l (Gibco BRL, Grand Island, NY, USA). HeLa^{3rd} subline was generated according to previous study [30]. HPV-immortalized cervical epithelial cells (Z172 and Z183A) were cultured in DMEM with 10% (w/v) Nu-Serum (BD Bioscience, San Jose, CA, USA) and hydrocortisone at 50 ng/ml (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Transfection and Sp1 and EZH2 overexpression

Cells were seeded in 24-well plates at 80%–90% confluence and then transfected with various amounts of pCDNA3.1-*Sp1*-V5 (NM_138473.2) and pCDNA3-3myc-*EZH2* (a gift from Dr. Cha's laboratory [38]). These expression plasmids and vector controls were transfected with the Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instruction.

2.3. Knockdown of Sp1 and EZH2 by RNA interference

Cells were transfected with plasmid containing short hairpin RNAs (shRNAs) of human *Sp1* that were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan. The target sequences for human *Sp1* shRNA #1 and shRNA #2 were 5′-CCCAAGTTTATTTCTCTCTTA-3′ (TRCN0000020444) and 5′-GGCAGATCTGCAGTCCATTAA-3′ (TRCN0000274153), respectively. A plasmid containing scrambled shRNA (LacZ) that targeted non-specific sequencing at 5′-TGTTCGCATTATCCGAACCAT-3′ (TRCN0000072223) was used as a negative control. In addition, short hairpin RNAs (shRNAs) of human EZH2 (GenBank: NM_004456) were purchased from Thermo Fisher Scientific (MA, USA). The target sequences of human EZH2 shRNA #1 and shRNA #2 were 5′-GAAAGAACGGAAATCTTAA-3′ (V2LHS_238994) and 5′-GAGGATCACCGAGATGATA-3′ (V2LHS_63068), respectively. A plasmid containing scrambled shRNA was used as a negative control.

2.4. Construction of Sp1 expression vector and LMX1A promoter

Human *Sp1* cDNA clone (BC062539.1) was purchased from Thermo Fisher Scientific (MA, USA). *Sp1* cDNA was generated by PCR with primer sets (Sp1+101-BamHIforward 5′-CTCGGATCCAGCGACCAAGATCACTC-3′ and Sp1+2437-XhoIreverse 5′-ACTCGAGTCAGAAGCCATTGCCACTG-3′) and constructed into a shuttle yT&A cloning vector (Yeastern Biotech Co., Ltd., Taipei, Taiwan). Created yT&A-*Sp1* plasmid was digested with BamHI and XhoI and then cloned into pCDNA3.1-V5 vector (Invitrogen) to generate pCDNA3.1-*Sp1*-V5 construct. The human *LMX1A* promoter (−1458/+221) was amplified by PCR with primer sets (*LMX1A*−1458 forward 5′-GAGTCCCATGGCCTCTGTAC-3′ and *LMX1A*+221 reverse 5′-GTTCGGGCCGGGCCGG-3′). The PCR product was constructed into a shuttle yT&A cloning vector. Created yT&A-*LMX1A*-p−1458/+221 plasmid was digested with KpnIand BglII and then cloned into pGL4.21 basic vector (Promega, Madison, WI, USA) to generate pGL4.21-*LMX1A*-p−1458/+221 construct.

2.5. Deletion and site-directed mutagenesis of the LMX1A promoter

Different deletion constructs of the *LMX1A* promoter were amplified by PCR with the same reverse primer *LMX1A*+221 and specific forward primer sets: 5′-CACGGGACGCGCTGCC-3′ for the *LMX1A*-p−1016/+221; 5′-CTCCGCAACTTCTCTCTGCT-3′ for the *LMX1A*-p−624/+221; 5′-GTATAGGTTGGGGCGGAGTC-3′ for the *LMX1A*-p−133/+221; 5′-GCAGGAGAAGGAGAAACGCAG-3′ for the *LMX1A*-p−62/+221, respectively. These PCR products were cloned into shuttle yT&A cloning vectors. Generated yT&A-*LMX1A*-promoter constructs were digested with KpnI and BglII and then constructed into pGL4.21 basic vector (Promega). The mutant constructs of *LMX1A* reporters were generated from yT&A-*LMX1A-p*−133/+221 plasmid using the Finnzymes Phusion® Hot Start High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., MA, USA) and mutant primers. Mutagenesis primers were as follows: Mut-Sp1-2F, forward, 5′-GTATAGGTTGTGTCTGAGTCGGATTC-3′ and complement strand Mut-Sp1-2R, 5′-CCGAATCCGACTCAGACACAACCTATACGA-3′; Mut-Sp1-1F, forward 5′-ACGCAGTTGTGTGTCGTAGGCCTAAGTAC-3′ and complement strand Mut-Sp1-1R, 5′-GTACTTAGGCCTACGACACACAACTGCGTT-3′. Generated mutant yT&A-*LMX1A*-p−133/+221 constructs were digested with KpnI and BglII and then cloned into pGL4.21 basic vector (Promega) to establish the mutant *LMX1A* reporter.