Reprogramming Antagonizes the Oncogenicity of HOXA13-Long Noncoding RNA HOTTIP Axis in Gastric Cancer Cells

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Abstract

Reprogramming of cancer cells into induced pluripotent stem cells (iPSCs) is a compelling idea for inhibiting oncogenesis, especially through modulation of homeobox proteins in this reprogramming process. We examined the role of various long noncoding RNAs (lncRNAs)-homebox protein HOXA13 axis on the switching of the oncogenic function of bone morphogenetic protein 7 (BMP7), which is significantly lost in the gastric cancer cell derived iPS-like cells (iPSLCs). BMP7 promoter activation occurred through the corecruitment of HOXA13, mixed-lineage leukemia 1 lysine N-methyltransferase, WD repeat-containing protein 5, and lncRNA HoxA transcript at the distal tip (HOTTIP) to commit the epigenetic changes to the trimethylation of lysine 4 on histone H3 in cancer cells. By contrast, HOXA13 inhibited BMP7 expression in iPSLCs via the corecruitment of HOXA13, enhancer of zeste homolog 2, Jumonji and AT rich interactive domain 2, and lncRNA HoxA transcript antisense RNA (HOTAIR) to various cis-element of the BMP7 promoter. Knockdown experiments demonstrated that HOTTIP contributed positively, but HOTAIR regulated negatively to HOXA13-mediated BMP7 expression in cancer cells and iPSCs, respectively. These findings indicate that the recruitment of HOXA13–HOTTIP and HOXA13–HOTAIR to different sites in the BMP7 promoter is crucial for the oncogenic fate of human gastric cells. Reprogramming with octamer-binding protein 4 and Jun dimerization protein 2 can inhibit tumorigenesis by switching off BMP7.

Introduction

The gene for homeobox protein HOXA13 (HOX13) is located as the most-posterior gene of the HOXA cluster in 7p15.2 [1]. Expression of HOXA13 is important for homeostasis in morphogenesis [1]. HOXA13 has a role in cancer promotion of liver stem-like cell lines [2] and the growth of esophageal cancer [3], and is involved in hepatocarcinogenesis [4] and leukemogenesis [5]. Moreover, HOXA13 is a poor prognostic marker for gastric cancer patients [6]. Recently we found that upregulation of HOXA13 plays a critical role in the
tumorigenesis of gastric cancer by comparing the nontumorigenic gastric cell line CSN and its derivative cancerous subline CS12 [7].

HOXA13 is reported to directly control the expression of bone morphogenetic protein 7 (BMP7) via binding to its enhancer element [8, 9]. BMP7 is a multifunctional growth factor belonging to the transforming growth factor beta superfamily. In addition to its function in bone formation and cartilage development, BMP7 is relevant in cell proliferation, apoptosis, and organ regulation and repair. In cancer, BMP7 behaves as an oncogene [10] or anti-oncogene [11] depending on ligands and cell types [12].

The long noncoding RNA (IncRNA) HOTAIR transcript at the distal tip (HOTTIP), located at the 5′-end of the HOXA cluster, is expressed significantly in anatomically distal human fibroblasts [13]. The interaction of the HOTTIP and WD repeat-containing protein 5 (WDR5)/mixed-lineage leukemia 1 lysine N-methyltransferase (MLL1 also known as histone-lysine N-methyltransferase 2A or KMT2A) in Tithorax complex enhances histone H3 lysine 4 trimethylation (H3K4me3) to activate the expression of 5′-HOXA genes including HOXA13 [14, 15]. Increased HOTTIP expression is a negative prognostic factor in some cancers [16] and promotes tumor progression and drug resistance by regulating HOXA13 in pancreatic cancer [17]. Another IncRNA HOTAIR is expressed from the posterior region of the HOXC11–12 cluster and is implicated in cancer development. HOTAIR interacts with enhancer of zeste homolog 2 (EZH2), suppressor of zeste 12 homolog subunit (SUZ12) in Polycomb repressive complex 2, and lysine demethylase 1A (LSD1 or KDM1A) to mediate repressive histone modifications at H3K27 and H3K4 at posterior HOXD locus [18, 19]. Thus, the actions of HOTTIP and HOTAIR to HOXA13 and HOXD10 loci are cis and trans, respectively [14, 15, 18, 19]. However, to our knowledge no common targets, which are regulated by both IncRNAs are present previously and the mechanistic regulation of the IncRNAs-HOX axis on their target remains to be clarified [20].

We have shown that the Jun dimerization protein 2 (JDP2) is a modulator of wingless-related integration site signaling pathway [21] and works together with octamer-binding protein 4 (OCT4) for the reprogramming of medulloblastoma to generate induced pluripotent stem cell (iPSC)-like cells (iPSCs) [22]. Liu et al. [23] also reported that JDP2 reprograms the somatic cells into the iPSCs through the five non-Yamanaka factors (Id1, Jhdhm1b, Lrh1, Sal14, and Glis1). Thus, JDP2 is a critical factor for the determination of stemness. Here, we used iPS technology [24] with JDP2 and OCT4 to reprogram CS12 gastric cancer cells to generate induced pluripotent cancer cells (iPSCs) [22]. Liu et al. [23] also reported that JDP2 reprograms the somatic cells into the iPSCs through the five non-Yamanaka factors (Id1, Jhdhm1b, Lrh1, Sal14, and Glis1). Thus, JDP2 is a critical factor for the determination of stemness. Here, we used iPS technology [24] with JDP2 and OCT4 to reprogram CS12 gastric cancer cells to generate induced pluripotent cancer cells [25, 26], and found a reduced tumorigenicity in CS12-derived iPSCs, suggesting that reprogramming of CS12 cells seems to abrogate the oncogenic function of HOXA13 in CS12 cells [7]. Mechanically, although the expression of HOXA13 was increased in both CS12 cells and iPSCs, HOXA13 behaved as an oncogene in CS12 but acted as a tumor suppressor in iPSCs through working with HOTTIP and HOTAIR to up- and down-regulate BMP7 expression, respectively. Knockdown of HOTTIP and BMP7 in CS12 cells decreased cancerous features but downregulation of HOTAIR in iPSCs promoted cell proliferation. These results suggest that networking between HOXA13 and IncRNAs can manipulate the oncogenic expression of BMP7 in human gastric cancer cells.

**MATERIALS AND METHODS**

**Cell Lines, Animals, Antibodies**

Human gastric normal cells CSN and cancerous CS12 cells were cultured as described elsewhere [27]. 293T, and mouse embryonic fibroblasts (MEFs) were obtained from the RIKEN Cell Bank (Tsukuba, Ibaraki, Japan, http://cell.brc.riken.jp/en/) and cultured in Dulbecco’s modified Eagle’s minimal essential medium (DMEM; Gibco, Carlsbad, CA, https://www.thermofisher.com) supplemented with 10% charcoal-stripped fetal bovine serum (FBS; Gibco) with or without 1% penicillin and streptomycin (Gibco) as described elsewhere [7]. The animal welfare guidelines for the care and use of laboratory animals were approved by the Animal Care Committee of the RIKEN Bioresource Center in Japan, the National Laboratory of Animal Center and the Kaohsiung Medical University in Taiwan. All antibodies used in this work are listed in Supporting Information Table S1.

**Plasmids, Small Interference RNA, and Short Hairpin RNA Lentivirus**

The human HOXA13 and BMP7 cDNAs were obtained from the RIKEN DNA Bank (IRAK168110; Tsukuba, Ibaraki, Japan, http://dna.brc.riken.jp/) and were inserted into pcDNA3 (ThermoFisher Scientific, Waltham, MA, https://www.thermofisher.com), pPyCAG-GFP, or pPyCAG-IP [27], to generate expression plasmids for cancer or stem cells. BMP7 promoter [−1,712 nucleotide (nt) to +110 nt] was obtained from ActiveMotif (NM000522.4; Carlsbad, CA, https://www.activemotif.com/) and was inserted into pGL4 luciferase vectors (Promega, Madison, WI, https://www.promega.com). The mutations of HOX binding sites (S1 to S3) at BMP7 promoter were generated by polymerase chain reaction using the primers of 5′-CTTGGAAATAGCGCAGGCGGTGGA-3′ (S1), 5′-GCATGTTAGCAGTTAGTGAGT-3′ (S2), and 5′-GTA CCGAGCTAGCTACTCCCACT-3′ (S3). All constructs were confirmed by DNA sequencing. The short hairpin RNA (shRNA) lentiviruses were generated in 293T cells cotransfected with pCA G-HIVgp-IN-D64V, pCMV-HSV-G-RSV-Rev, or pCMV-dr8.91, or pCMV-d8.74pSPAX2 or VSVG/pMD2G, and TRCnn000355645 or TRCnn000355646 (c-JUN), TRCnn000015405 or TRCnn000015406 (HOXA13, Academia Sinica, Taipei, Taiwan, http://rna.genmed.sinica.edu.tw), PLKO.1-GFP (No. 30323; Addgene, Cambridge, MA, https://www.addgene.org) or scrambled control shRNA (Sigma-Aldrich, St. Louis, MO, https://www.sigmaaldrich.com) [7]. Virus was purified at 72 hours after post-transfection as described [28]. The CS12 cells (1 × 10⁵) were infected with virus at a multiplicity of infection (MOI) of 2 or 4. After cultivation for 3 days, cells were injected into severe combined immunodeficiency (SCID) mice (5 × 10⁶ cells/spleen) as described elsewhere [29]. Lentiviruses for human BMP7-shRNA (SV-39748) and HOXA13-shRNA (TR312365, TR312365) were obtained from Santa Cruz Biotech. (Dallas, TX, https://www.scbt.com) and OriGene (Rockville, MD, http://www.origene.com). For small interference RNA (siRNA)-mediated gene knockdown, cells were transfected with siRNA (ThermoFisher Scientific) against BMP7 (s2035, s2036, s2037), HOXA13 (s6785, s6786, s6787), HOTTIP (n509686, n509687), HOTAIR (n272221, n272230), c-JUN (s7659) [29], HOTAIRM1 (n507509, n507510), or negative control pool (s43418, s43420; Dharmaco, D-001810–10–05) using Lipofectamine RNA iMAX reagents (ThermoFisher Scientific). The effects on gene silencing were examined after 48 hours of incubation.
Generation of iPSLCs From Human Gastric Cancer Cell Line CS12

The CS12 gastric cancer cells (5 \times 10^5) were transfected with OCT4 and JDP2 by electroporation as described elsewhere [30, 31]. The cells were then selected with G418 (100 \mu g/ml) for 2 days, and replated onto mitomycin C-treated MEFs in the standard iPS medium (DMEM [Gibco] supplemented with 10% FBS and antimycotics-antibiotics [AM-AB; Gibco] plus human leukemia inhibitory factor [LIF; 10 ng/mL, ThermoFishier Scientific]) [29, 31] for 2–3 weeks to obtain reprogrammed iPSLCs. Alternatively, recombinant lentiviruses CSIV-CMV-MCS-IRES2-Venus-encoding human OCT4 and JDP2 were generated using 293T cells. CS12 cells (5 \times 10^5) were infected with the lentiviruses (MOI = 2) and incubated for 1 week in iPS medium with LIF as described above. Thereafter, cells were transferred onto mitomycin C-treated MEFs in iPS medium plus human LIF (10 ng/ml) to obtain reprogrammed iPSLCs.

Cell Proliferation, BrdU-Incorporation, and Colony Assay

The numbers of living cells were quantitated using a trypsin blue dye-exclusion assay [21, 29]. To analyze the cell cycle, serum-starved cells were stimulated with 15% FBS and then labeled with 10 \mu M BrdU (Sigma-Aldrich) for 1 hour, trypsinized and fixed overnight with 70% ethanol at 4°C. The fixed cells were incubated in 2M HCl for 1 hour at room temperature, neutralized with 0.1 M Na_2B_4O_7(OH)_2 \cdot 8H_2O, stained by BrdU antibody (BD Bioscience, San Jose, CA, http://wwwbdbiosciences.com) at room temperature for 2 hours, followed by Alexa 488-conjugated secondary antibody at room temperature for 30 minutes. DNA was stained with propidium iodide (PI; 5 \mu g/ml). Cell samples were analyzed with EPICS XL-MCL flow cytometer (Beckman Coulter, Miami, FL, https://wwwbeckmancoulter.com). To quantitate the cells in S phase, the BrdU Labeling and Detection Kit (Sigma-Aldrich) was used [29]. For the colony-formation assay, cells were plated in duplicate at 5 \times 10^3 cells/10 cm gelatin-coated dish. Two weeks later, colonies with a diameter > 2 mm were counted after staining with Giemsa staining solution (Wako Chemicals, Tokyo, Japan, http://www.wako-chem.co.jp/english/).

Teratoma, Tumor Formation, and Immunohistochemistry

CS12 and CS12/iPSLCs were injected subcutaneously into the dorsal flank of SCID mice as described [7, 29]. Tumor size was estimated once or twice a week using a caliper. Tumor volume was calculated according to the formula: volume (cm^3) = (L \times W^2)/2, where L and W are the length and width of the tumor, respectively. The teratomas were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Alkaline Phosphatase, Immunocytochemistry, and Karyotyping

Alkaline phosphatase activity measurement and immunocytochemistry were performed as described elsewhere [29, 30]. Chromosome spreads for karyotyping were prepared using a conventional air-drying technique. GTG (G-banding) staining was performed as described elsewhere [32].

Migration, Invasion, and Chemoresistance Assays

Cells (1 \times 10^4 cells) resuspended in DMEM without FBS were seeded in the upper Transwell with or without Matrigel (1 mg/ml, Corning, NY, https://www.corning.com) as described elsewhere [29]. The lower plate contained DMEM plus 10% FBS. Three days later, the cells moved to downside of Transwell were fixed with 4% formaldehyde, stained with 1% crystal violet, and counted under a microscope. Regarding chemoresistance assay, cells were seeded in 96-well plates for 24 hours and then treated with a serial mixture of cisplatin, 5-fluorouracil, and doxorubicin for additional 48 hours. Cell viability was examined using the MTT assay as described elsewhere [21, 29].

Western Blotting

Western blotting was conducted as described elsewhere [7, 21]. Briefly, cell lysates were prepared in SDS sample buffer or Triton X-100 lysis buffer (10 mM HEPES, pH 7.8, 5% glycerol, 1% Triton X-100, 5 mM EDTA, 50 mM NaF, 20 mM β-glycerophosphate, 50 μg/ml aprotinin, 100 μg/ml leupeptin, 25 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM PMSF), subjected to SDS–PAGE, and electrotransferred onto a polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA, https://www.merckmillipore.com). After antibody incubation, ECL immunodetection was performed. Results were analyzed using a ChemiDoc XRPlus analyzer (Bio-Rad, Hercules, CA, http://www.bio-rad.com).

Reverse Transcriptase PCR and Quantitative Polymerase Chain Reaction

Total RNA was extracted from cells using the TRIzol reagent (ThermoFisher Scientific). RNA was reverse transcribed to cDNA using a reverse transcription kit (Promega). PCR was performed using the GoTaq Green Master Mix (Promega). Quantitative polymerase chain reaction (qPCR) was performed using the QuantiFast SYBR Green PCR kit (Qiagen, Gaithersburg, MD, https://www.qiagen.com) as described elsewhere [7, 21]. β-actin and GAPDH were used as internal controls. Primer sequences are listed in Supporting Information Tables S2–S4.

Transient Transfection and Luciferase Assay

Transient transfection and the luciferase assay were performed as described elsewhere [7, 21, 33–35]. Cells were plated into each well of a 12-well plate and cultured for 24 hours. The cells were then cotransfected with indicated amounts of various effector constructs and BMP7-promoter-luciferase reporters using Lipofectamine 2000 (Invitrogen). The total amount of transfected DNA was kept constant at 1 μg per well by the addition of pBluescript. After 48 hours or the indicated period of incubation, cells were harvested for luciferase activity assay using Dual-Luciferase Reporter Assay System (Promega) and an illuminometer (Berthold Technologies, Bad Wildbad, Germany, https://www.berthold.com).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was performed as described elsewhere [7, 29, 33]. The immunoprecipitated protein–DNA complexes were washed twice with binding buffer (10 mM HEPES, pH 7.9, 10 mM Tris–HCl, pH 7.9, 12.5% glycerol, 0.25% NP-40, 0.5% Triton X-100, 0.24 M NaCl, 1 mM MgCl_2, 0.5 mM EDTA, and 1% Triton X-100), resuspended in SDS sample buffer, and incubated at 4°C for 2 hours. The samples were then centrifuged, and the supernatants were used for ChIP assay as described elsewhere [7].
0.75 mM MgCl₂, 1.1 mM EDTA, and protease inhibitor mixture) and then washed twice with Tris-EDTA buffer (10 mM Tris-HCl, pH 7.9, and 1 mM EDTA). The protein–DNA complexes were disrupted with proteinase K (Sigma-Aldrich) at pH 6.8. DNA was extracted with phenol and chloroform, precipitated in ethanol, and analyzed by real-time PCR using the Power SYBR Green Master Mix (Invitrogen). The PCR conditions consisted of 1 cycle of 2 minutes at 50°C and 1 cycle of 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds, and 55°C–60°C for 60 seconds. The primers used in these experiments are shown in Supporting Information Table S5.

RNA Immunoprecipitation Assay
RNA immunoprecipitation (RIP) assay was performed as described elsewhere [36]. Cells were lysed in RIP lysis buffer (10 mM HEPES, pH 8.0, 40 mM KCl, 3 mM MgCl₂, 5% glycerol, 2 mM DTT, 0.5% sodium deoxycholate, 100 unit/ml RNase inhibitor and protease inhibitors) and the resulted cell lysates (1 mg) were incubated with HOXA13 antibody for 2 hours, followed by 1 mg/ml RNA-precleared protein A/G beads for another 2 hours. The immunoprecipitates were washed by RIP lysis buffer and resuspended in TRIzol reagent (ThermoFisher Scientific) to extract the immunoprecipitated RNA for reverse transcription and qPCR.

Statistical Analyses
The data are presented as the mean ± SEM from 3–6 experiments. Difference between two and multiple experimental groups was assessed using two-tailed Student’s t tests and one-way ANOVA, respectively. Survival analysis was performed using the Kaplan–Meier method with log-rank test. p < .05 was considered statistically significant.

RESULTS
Comparative Expression of Stemness Genes in iPSLCs and CS12 Cells
We used transcription factors, JDP2 and OCT4, to induce reprogramming and generate iPSCs as described previously [22], because it was not feasible to use the standard four Yamanaka factors [24] to generate stem cell-like colonies (data not shown) in the normal human gastric mucosa cell line CSN and its derived CS12 cancer cells [7]. Three weeks after electroporation of JDP2 and OCT4, small, packed, domed iPSC-like colonies were detected (Fig. 1A). The number of colonies with a typical iPSC phenotype increased over time by repetitive passage. When iPSCs were cultured for more than 4 weeks, the CS12iPSCs displayed strong alkaline phosphatase activity (Fig. 1A). qPCR revealed that the endogenous expression of OCT4 and KLF4 in CS12iPSCs increased about 3.4- and 5.3-fold, respectively, but NANOG and ESRRB were repressed by 50% compared with CS12 cells (Fig. 1B). Endogenous expression of JDP2 and p53 was also reduced by about 50% in CS12iPSCs (Fig. 1B). RNA sequence analysis showed that the primitive germ layer markers such as SOX1 (ectoderm), SOX17 (endoderm), and T (mesoderm) were not changed between CS12 and CS12iPSCs (Supporting Information Fig. S1A); by contrast, the pluripotent markers ESRRB and KLF2, but not REX1 and SALL4, were reduced by 30%–45% in CS12iPSCs (Supporting Information Fig. S1A). Immunofluorescence staining confirmed the expression of stemness markers (OCT4, SOX2, NANOG, SSEA4, Tra-1–60, Tra-1–81) in CS12iPSCs (Fig. 1C). To confirm the pluripotency [29], we transferred CS12iPSCs into SCID mice to generate teratomas, which showed two germ layers such as endoderm and ectoderm (Fig. 1D). However, three germ layers were not observed in any instance. These results suggest that CS12iPSCs were not fully pluripotent. The karyotype analysis showed no changes of chromosomes in CS12iPSCs (Supporting Information Fig. S1B).

CS12iPSCs Exhibited Reduced Tumorigenicity In Vivo
To compare the tumorigenicity of CS12 and CS12iPSCs, we injected these cells subcutaneously into SCID mice. One month after injection, tumors generated by CS12 cells expanded to 2.5 cm³, which was 10 times the size of those generated by CS12iPSCs (Fig. 1E). Both sizes and weights of tumors generated by CS12iPSCs were smaller than those of CS12 cells (Fig. 1F, 1G). CS12iPSCs-derived xenografts exhibited reduced numbers of necrotic cells and the cells with large nucleus and mitosis (Fig. 1F). Thus, CS12iPSCs exhibited reduced tumorigenicity in vivo when compared with CS12 cells.

Decreased Cell Proliferation, Colony Formation, Cell Migration and Invasion, and Drug Resistance in CS12iPSCs
To verify the difference in oncogenic activity between CS12 and CS12iPSCs, cell proliferation, colony formation, cell migration, invasion, and drug resistance were examined. The CS12 cells proliferated more rapidly than CS12iPSCs and CSN cells (Fig. 2A). The colony-forming ability of CS12 cells was twofold higher than that of CS12iPSCs (Fig. 2B). Cell cycle analysis of bromodeoxyuridine (BrdU)-labeled cells and BrdU ELISA assay confirmed that cell proliferation of CS12iPSCs was reduced (Fig. 2C). No increase in apoptosis was found in CS12iPSCs (Supporting Information Fig. S1C). Transwell assays demonstrated that both the migration and invasion efficacies of CS12 cells were higher than those of CS12iPSCs and CSN cells (Fig. 2D), which were consistent with the increased expression of Snail 1 family transcriptional repressor 1/2 (Snail1/2), Matrix metalloproteinase 2 (MMP2), Vimentin, and with the decrease of E-CADHERIN in CS12 cells (Supporting Information Fig. S1D, S1E). To examine the drug resistance, cell viability was measured by incubating cells with a mixture of three conventional drugs for chemotherapy: 5-fluorouracil, cisplatin, and doxorubicin. CS12 cells showed greater resistance to these drugs than CS12iPSC and CSN cells (Fig. 2E), demonstrating the increased chemoresistance of CS12 cells.

Enhanced Expression of HOXA13 in CS12iPSCs and CS12 Cells
We previously reported that several HOXA genes were upregulated in CS12 when compared with CSN cells [37] and upregulated HOXA13 contributed to the tumorigenicity of CS12 cells [7]. Here, we found that the expressions of HOXA4, 5, 7, and 9 were decreased in CS12iPSCs when compared with
Figure 1. Generation and tumorigenicity of iPSLCs from CS12 gastric cancer cells. (A): Schematic representation of iPSLCs generation from CS12 cells by OCT4 and JDP2. Morphology (CS12 and CS12/iPSLCs) and alkaline phosphatase staining (CS12/iPSLCs) are shown (magnification, ×100). (B): Expression of stemness genes analyzed by quantitative reverse transcriptase PCR (RT-PCR). The plot shows the mean (n = 5) ± SEM (*, p < .05; **, p < .01). (C): Immunocytochemical analysis of pluripotency markers in CS12/iPSLCs (magnification, ×200). (D): (Left panel) Pluripotency of CS12/iPSLCs. Teratomas that were generated by the transplantation of CS12/iPSLCs into severe combined immunodeficiency (SCID) mice were examined by immunohistochemistry, which shows differentiation of the ectoderm (keratinocytes) and endoderm (glandular cells). White bar, 100 μm. (Right panel) Expression of differentiation marker genes were examined by quantitative RT-PCR. The plot shows the mean (n = 5) ± SEM (*, p < .05). (E): Tumor formation of subcutaneously injected CS12 and CS12/iPSLCs in SCID mice. Tumor was sectioned and stained with hematoxylin and eosin (scale bar, 50 μm). (F): Tumor sizes of CS12 and CS12/iPSLCs. The plot shows the mean (n = 5) ± SEM (**, p < .01). (G): Tumor weight of CS12 and CS12/iPSLCs. Plot shows the mean (n = 5) ± SEM (**, p < .01). Abbreviations: MEFs, mouse embryonic fibroblasts iPS, induced pluripotent stem; LIF, leukemia inhibitory factor; iPSLCs, induced pluripotent stem cell-like cells; JDP2, Jun dimerization protein 2; OCT4, octamer-binding protein 4.
CS12 cells (Fig. 3A; Supporting Information Figure S2A). Unexpectedly, HOXA13 was further increased in CS12iPSLCs (Fig. 3A, 3B; Supporting Information Figure S2A), implying that other factors might be involved in determining the oncogenic activity of HOXA13.

To clarify the effects of HOXA13 on the different tumorigenicity between CS12 and CS12iPSLCs, HOXA13-knockdown CS12 cells (Fig. 3C), and CS12iPSLCs (Fig. 3E) were inoculated into SCID mice to compare xenograft formation ability. Knockdown of HOXA13 in CS12 cells decreased tumor...
Figure 3. Role of HOXA13 in tumor formation of CS12 and CS12/iPSLCs xenografts. (A): Comparative mRNA expressions of HOXA family genes in CS12 and CS12/iPSLCs were examined by quantitative reverse transcriptase PCR. The data are presented as mean ± SEM. Relative expression of HOXA mRNA in CS12 cells was set as one. (B): Comparative expression of HOXA13 proteins in CSN, CS12, and CS12/iPSLCs. The exposure time is 100 seconds. Data are presented as means (n = 3) ± SEM, *, p < .05. (C): Expression of HOXA13 was examined by Western blot in CS12 cells treated with siRNA against HOXA13, c-JUN, or scramble control. The exposure time was 50 seconds. (D): The effect of HOXA13 knockdown on tumor growth of CS12 xenografts. (E): Comparative expression of HOXA13 was examined by Western blot in CS12/iPSLCs treated with siRNA against HOXA13, c-JUN, or scramble control. The exposure time is 50 seconds. (F): The effects of HOXA13 knockdown on tumor growth of CS12/iPSLCs xenografts. (G, H): The effects of HOXA13 knockdown on tumor sizes of CS12 (G) and CS12/iPSLCs (H) xenografts. (I, J): The effects of HOXA13 knockdown on tumor weights of CS12 (I) and CS12/iPSLCs (J) xenografts. All data in (G–J) were derived from six independent experiments and are presented as mean ± SEM (**, p < .01). Abbreviation: iPSLCs, induced pluripotent stem cell-like cells.
formation and tumor weight (Fig. 3D, 3G, 3I). In contrast, HOXA13-knockdown CS12iPSLCs increased tumor size and tumor weight (Fig. 3F, 3H, 3J). These data support the notion that HOXA13 in conjunction with some unspecified factors determined the tumorigenicity of CS12 cells and CS12iPSLCs.

**Differential Expressions of BMP7 and IncRNAs were Critical to Determine the Tumorigenicity of CS12 Cells and CS12iPSLCs**

To further characterize the differences between CS12 and CS12iPSLCs, we examined the expression profiles of CS12 and CS12iPSLCs using RNA sequencing and confirmed that BMP7, a known HOXA13 target [8, 9], was upregulated in CS12 compared with that in CS12iPSLCs (Fig. 4A; Supporting Information Fig. S3B). Expressions of BMP7 protein and phosphorlated SMAD1 protein were indeed upregulated in CS12, suggesting the crossstalk of BMP7-SMAD axis [9] (Supporting Information Fig. S2B). BMP7 might be critical because it was altered significantly in several gene ontology categories, such as stem cancer stem, pluripotent stem progenitors, epithelial-mesenchymal transition (EMT)/mesenchymal-epithelial transition (MET), cytokines, and growth factors (Supporting Information Fig. S3B). Knockdown of HOXA13 but not c-JUN (as a control) in CS12 cells decreased BMP7 protein level (Supporting Information Fig. S3A). Expressions of other HOXA13 targets including BMP2, insulin-like growth factor-binding protein 3 and annexin A2 as well as FGF8 were not changed (Fig. 4A).

We also found the preferential upregulation of HOTTIP in CS12 cells and HOTAIR in CS12iPSLCs, respectively (Fig. 4B). The expression of another HOXA transcript antisense RNA, myeloid-specific 1 (HOTAIRM1) expression was unchanged (Fig. 4B). Because HOTTIP and HOTAIR regulate expression of HOX gene clusters, and HOXA13 controls BMP7 expression, we next examine the role of HOTTIP/HOTAIR–HOXA13–BMP7 axis in tumorigenicity by knockdown experiments (Fig. 4C, 4D).

In CS12 cells, siHOTTIP reduced the expression of HOXA13 as reported [6, 12, 14], but siHOTAIR did not affect the expression of HOXA13. By contrast, in CS12iPSLCs, siHOTTIP did not affect the expression of HOXA13, but siHOTAIR decreased the expression of HOXA13 (Supporting Information Fig. S4A). In CS12 cells, knockdown of BMP7 and HOTTIP, but not HOTAIR, decreased cell growth, colony formation, cell migration and invasion activity (Fig. 4E–4H), suggesting that HOTTIP and BMP7 contribute to the tumorigenicity of CS12 cells. Conversely, knockdown of HOTAIR, but not BMP7, in CS12iPSLCs, increased cell growth, colony formation, cell migration, and invasion activity (Fig. 4E–4H), indicating that HOTAIR played a tumor suppressor role in CS12iPSLCs. Knockdown of HOXA13 resulted in a reduced cell growth in CS12 cells but caused an increase of cell proliferation in CS12iPSLCs (Fig. 4E), which was consistent with the results of mouse xenograft model (Fig. 3). The reduced tumorigenicity of CS12iPSLCs was also associated with restored expressions of p53 and several CDK inhibitors including p16\textsuperscript{ink4a}, p15\textsuperscript{ink4b}, p18\textsuperscript{inn}, p21\textsuperscript{Cip1}, p27\textsuperscript{Kip2}, and p53\textsuperscript{Kip2}, together with decreased E2F-1 expression in CS12iPSLCs compared with that in CS12 cells (Supporting Information Fig. S4B).

On the other hand, overexpression of BMP7 in CS12iPSLCs increased cell proliferation, colony formation, and cell migration and invasion (Supporting Information Fig. S5A–S5E). The differential recruitment of HOXA13 to the BMP7 promoter at S1 and S2 Sites in CS12 and CS12iPSLCs, Respectively

Given that BMP7 was differentially expressed in CS12 and CS12iPSLCs (Fig. 4A, Supporting Information Fig. S2B, S3B), and was critical for determining the tumorigenicity of both cells (Fig. 4E–4H), and forced expression of BMP7 enhanced tumorigenic features in CS12iPSLCs (Supporting Information Fig. S5), we investigated how BMP7 expression was regulated in both cells. It is known that HOXA13 directly binds to the BMP7 promoter and induces BMP7 expression [9, 38, 39]. We conducted ChIP assays to examine the binding of HOXA13 to the BMP7 promoter in CS12iPSLCs and CS12 cells. Computational sequence analysis revealed three putative HOX-binding sites: S1 (~−412/~416), S2 (~−1,113/~1,117), and S3 (~−1,613/~1,617) in the BMP7 promoter (Fig. 5A) (PROMO3; TRANSFAC v8.3) [40]. The results of ChIP showed that recruitment of HOXA13 to the S1 site was increased in CS12 cells but not in CS12iPSLCs. By contrast, binding of HOXA13 to the S2 site was increased in CS12iPSLCs but not in CS12 cells (Fig. 5A). No significant recruitment of HOXA13, HOTA9, or HOX7 was found at the S3 site and nonspecific NS site. In addition, ChIP assays demonstrated an elevated trimethylation of lysine 4 on histone H3 (H3K4me3) and an increased recruitment of MLL1/ WDR5 at the S1 site in CS12 cells, but not in CS12iPSLCs (Fig. 5B), indicative of activated BMP7 promoter in CS12 cells. By contrast, increased H3K27me3 and recruitments of EZH2 and jumonji AT-rich interactive domain 2 (JARID2) [41] were found at the S2 site in CS12iPSLCs, but not in CS12 cells (Fig. 5B), explaining the reduced BMP7 expression in CS12iPSLCs. No significant difference in these epigenetic marks was observed at the S3 and NS sites in both cells (Fig. 5B). The expression levels of Polycomb group RING finger complex protein B lymphoma Mo-MLV insertion region 1 homolog (BMI1), EZH2, SUZ12, and JARID2 were significantly higher in CS12iPSLCs than in CS12 cells (Supporting Information Fig. S6). Moreover, RNA immunoprecipitation experiment [36] demonstrated that the association between HOXA13 and HOTTIP was predominant in CS12; by contrast, HOTAIR–HOXA13 interaction is evident in CS12iPSLCs (Fig. 5C). These findings suggested that the differential recruitment of HOXA13 was correlated with contrasting epigenetic marks at the S1 and S2 sites of the BMP7 promoter and conversed expression patterns of BMP7 in CS12 cells and CS12iPSLCs.

**HOXA13 Differentially Upregulates and Downregulates BMP7 Promoter Activity Through the S1 and S2 Sites, Respectively**

To further examine the roles of S1 and S2 sites in HOXA13-mediated BMP7 expression, we cloned BMP7 promoters, both wild-type and HOX-binding site mutants, into a luciferase reporter plasmid (Fig. 6A) and then cotransfected them with a HOXA13-expressing plasmid into CS12 cells. The results showed that HOXA13 transactivated wild-type and S2-
Figure 4. Identification of BMP7 and long noncoding RNAs as critical determinants of tumorigenicity. (A): Comparative mRNA expression of FGF8, BMP2, BMP7, IGFBP-3, ANXA2, and IL-6 in CSN, CS12, and CS12 iPSLCs was examined by quantitative reverse transcriptase PCR (RT-PCR). (B): Comparative expression of HOTTIP, HOTAIR, and HOTAIRM1 in CSN, CS12, and CS12iPSLCs was examined by quantitative RT-PCR. (C): Knockdown of GFP, HOTTIP, or HOTAIR by respective siRNAs in CS12 cells (left) and CS12iPSLCs (right). (D): Knockdown of BMP7 was examined by Western blot in CS12 (left) and CS12iPSLCs (right). The exposure time is 100 seconds (left) and 200 seconds (right), respectively. (E): Effect of siRNA against HOXA13, HOTTIP, HOTAIR, or BMP7 on cell proliferation of CS12 (left) and CS12iPSLCs (right) was examined by trypan blue dye-exclusion test. (F): Effect of siRNA against HOTTIP, HOTAIR, or BMP7 on colony formation of CS12 (left) and CS12iPSLCs. (G): Effect of siRNA against HOTTIP, HOTAIR, or BMP7 on cell migration of CS12 (left) and CS12iPSLCs (right). (H): Effect of siRNA against HOTTIP, HOTAIR, or BMP7 on cell invasion of CS12 (left) and CS12iPSLCs (right). All data are presented as mean ± SEM (n = 5; *, p < .05; **, p < .01). Abbreviations: BMP7, bone morphogenetic protein 7; HOTTIP, HoxA transcript at the distal tip; HOTAIR, HoxA transcript antisense RNA; iPSLCs, induced pluripotent stem cell-like cells.
mutated BMP7 promoters comparably, but just marginally induced S1-mutated BMP7 promoter (Fig. 6B), supporting that HOXA13 induced BMP7 expression through S1 site in CS12 cells. To examine the effect of HOXA13 on BMP7 promoter in iPSLCs, we used a feasible promoter (pPyCAG) to drive HOXA13 expression in stem cells [35]. The results showed that
only S2 mutant displayed increased activity but all other mutants and wild type BMP7 promoter constructs showed significant repression in CS12iPSLCs (Fig. 6C), suggesting that HOXA13 inhibited BMP7 expression through S2 site.

HOTTIP– and HOTAIR–Differentially Regulated BMP7 Promoter Activity and the Epigenetic Configurations at S1 and S2 Sites

Because HOTTIP and HOTAIR regulate gene expression through MLL–WDR5 and PRC2–EZH2 complexes, respectively [12, 17, 42], we examined the effects of siHOTTIP and siHOTAIR on the recruitments of MLL–WDR5 and SUZ–EZH2–JARID2 to the BMP7 promoter in CS12 and CS12iPSLCs. The results showed that knockdown of HOTTIP, but not HOTAIR, reduced H3K4me3 and decreased the recruitment of MLL/ WDR5 to the S1 site in CS12 cells (Fig. 7A), suggesting that HOTTIP knockdown might decrease BMP7 promoter activity. Knockdown of HOTAIR, but not HOTTIP, reduced H3K27me3 and decreased the recruitment of EZH2 and JARID2 to the S2 site of the BMP7 promoter in CS12iPSLCs (Fig. 7B), indicating that HOTAIR contributed to the suppressive configuration at the S2 site. The knockdown of HOXA13 also showed similar patterns of HOTTIP–MLL–WDR5 axis in CS12 cells and HOTAIR–EZH2–JARID2 axis in CS12iPSLCs (Fig. 7A, 7B). Indeed, knockdown of HOTTIP or HOXA13 decreased BMP7 promoter activity in CS12 cells (Fig. 7C), while HOTAIR knockdown increased BMP7 promoter activity in CS12iPSLCs (Fig. 7D). These findings demonstrated that HOTTIP upregulated BMP7 expression in CS12, but HOTAIR suppressed BMP7 expression in CS12iPSLCs, both were involved with HOXA13 (Fig. 7E).

DISCUSSION

In this study, we reprogrammed the human gastric cancer cell line CS12 using OCT4 and JDP2 to generate CS12iPSLCs (Fig. 1). This technique is useful for cellular reprogramming of somatic cells and cancer cells into stem cells or stem-like cells, which can serve as disease model or a platform for drug screening. Notably, the reprogrammed CS12iPSLCs exhibited less tumor formation capacity in SCID mice when compared with the parental CS12 cells (Fig. 1E–1G), suggesting that reprogramming of cancer cells may be a strategy that could be developed for the future therapy.

Accumulating evidence reveals that high-level expression of BMP7 correlates with increased invasion and metastasis.
Figure 7. Regulation of epigenetic configuration and BMP7 promoter activity by siRNA against HOTTIP, HOTAIR, and HOXA13. (A, B): Effect of siRNA against GFP, HOTTIP, HOTAIR, and HOXA13 on histone methylations and recruitments of WDR5-MLL1, and PRC2 complexes to the S1 and NS sites in CS12 cells (A) or to the S2 site and NS sites in CS12iPSLCs (B). (C, D): Effects of siHOTTIP, siHOTAIR, and siHOXA13 on BMP7 promoter activities in CS12 (C) and CS12iPSLCs (D), respectively. The untreated BMP7 promoter activity in CS12 cells was assigned as one. The data are presented as mean ± SEM (n = 5; **, p < .01). (E): Schematic representation of the role of HOXA13-HOTTIP axis and HOXA13-HOTAIR axis on the BMP7 promoter in CS12 and CS12iPSLCs. K4me3; histone H3K4me3, K27me3; histone H3K27me3. Trithorax complex is composed of MLL, WDR and other components including JARID2. Polycomb repressive complex 2 is composed of SUZ12, EZH2 and other components. Abbreviations: BMP7, bone morphogenetic protein 7; HOTAIR, HoxA transcript antisense RNA; HOTTIP, HoxA transcript at the distal tip; OCT4, octamer-binding protein 4.
[43], cartilage and bone repair [44], programmed cell death [45], and various cancers including gastric cancer [43, 46]. Our knockdown experiments also supported an essential role of BMP7 in promoting cell proliferation, migration, invasion, and colony formation in CS12 cells (Fig. 4E–4H). Moreover, we found that forced expression of BMP7 in CS12iPSLCs, whose BMP7 level was low, increased oncogenic phenotypes such as xenograft tumor formation in SCID mice, invasive and migratory activity, colony formation and cell proliferation (Supporting Information Fig. S5). These results indicate that, at least in gastric cancer, BMP7 behaves as an oncogene.

Our results demonstrated the importance of the HOTTIP/HOTAIR–BMP7 axis in gastric cancer (Fig. 4E–4H). In particular, the increase of tumorigenicity in CS12iPSLCs by HOTAIR knockdown suggests that HOTAIR is a tumor suppressor gene as previously reported in glioblastoma and pediatric ependymoma [47, 48]. However, some studies suggest that HOTAIR is more likely to be an oncogene [42, 49, 50]. Thus, the precise role of HOTAIR may be context-dependent.

The differential recruitment of HOXA13 at positive and negative regulatory sites of the BMP7 promoter is a new finding, which demonstrates that HOXA13 can play either a positive or a negative role in expression of the same gene. Most previous studies only show the oncogenic role of HOXA13. Our study reveals that HOXA13 has an anti-oncogenic activity in CS12iPSLCs possibly through suppressing BMP7 expression (Figs. 3F, 3H, 3J, 6C, 7D). This study also demonstrates a novel, opposing regulation of the BMP7 expression pattern, which is regulated by HOTTIP- and HOTAIR-mediated epigenetic changes at the S1 and S2 sites of BMP7 promoter, respectively (Fig. 7). These epigenetic changes are differentially regulated in CS12 and CS12iPSLCs, which represent different gastric cancer stages. Thus, the present study reveals the complexity of epigenetic regulation of BMP7 expression during the development of gastric cancer.

This study has identified the differential effects of HOTTIP and HOTAIR on the S1 and S2 sites of BMP7 promoter that is correlated with upregulated and downregulated BMP7 expression, respectively. However, this finding does not fully explain the differential HOXA13 binding of S1 and S2 sites that is also correlated with BMP7 expression. Several issues need further investigation. First, the recruitment of HOXA13 at the S1 and S2 sites after knockdown of HOTTIP and HOTAIR can be examined to show the effect of lncRNAs on HOXA13 recruitment to the BMP7 promoter. Second, whether HOTTIP and HOTAIR directly target to S1 and S2 sites, respectively, or HOTTIP and HOTAIR regulate epigenetic changes at S1 and S2 sites indirectly is still unclear. Although our data showed the increased recruitment of WDR5/MLL to the HOTTIP-regulated S1 site and EZH2/JARID2 to the HOTAIR-regulated S2 site (Fig. 5), we did not yet check the presence of HOTTIP and HOTAIR at these sites. Third, if HOTTIP and HOTAIR are recruited to the S1 and S2 sites, is HOXA13 involved in this recruitment?

Moreover, is HOXA13 required for HOTTIP and HOTAIR recruitment? The interactions between HOXA13 and HOTTIP–HOTAIR, either directly or through other factors such as WDR5 and EZH2/SUZ12, remain to be examined, although they are associated each other (Fig. 5C). We are exploring these questions to elucidate more comprehensively the mechanism underlying BMP7 expression regulated by HOXA13–HOTTIP–HOTAIR in different settings.

**Conclusion**

Here, we have identified BMP7 as the key target of HOXA13 to control cell growth, invasion, and migration of human gastric cancer cells. In addition to BMP7, the finding of involvement of the histone methyltransferase (HMT) MLL and EZH2 in regulating BMP7 expression has revealed potential targets for cancer therapy. Several HMT inhibitors are under development to inhibit MLL and EZH2-SUZ12 for therapeutic purpose. Thus, the MLL–WDR5–HOTTIP–HOXA13 axis in CS12 cells can be used to test the therapeutic efficacy of HMT inhibitors. Furthermore, the EZH2–JARID2–HOTAIR–HOXA13 axis in CS12iPSLCs provides a platform to screen carcinogens that increase BMP7 expression.

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**Author Contributions**

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**Disclosure of Potential Conflicts of Interest**

The authors indicated no potential conflicts of interest.

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