Depletion of Embryonic Stem Cell Signature by Histone Deacetylase Inhibitor in NCCIT Cells: Involvement of Nanog Suppression

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Abstract

The embryonic stem cell-like gene expression signature has been shown to be associated with poorly differentiated aggressive human tumors and has attracted great attention as a potential target for future cancer therapies. Here, we investigate the potential of the embryonic stem cell signature as molecular target for the therapy and the strategy to suppress the embryonic stem cell signature. The core stemness gene Nanog is abnormally overexpressed in human embryonic carcinoma NCCIT cells showing gene expression profiles similar to embryonic stem cells. Down-regulation of the gene by either small interfering RNAs targeting Nanog or histone deacetylase inhibitor apicidin causes reversion of expression pattern of embryonic stem cell signature including Oct4, Sox2, and their target genes, leading to cell cycle arrest, inhibition of colony formation in soft agar, and induction of differentiation into all three germ layers. These effects are antagonized by reintroduction of Nanog. Interestingly, embryonic carcinoma cells (NCCIT, NTERA2, and P19) exhibit a higher sensitivity to apicidin in down-regulation of Nanog compared with embryonic stem cells. Furthermore, the down-regulation of Nanog expression by apicidin is mediated by a coordinated change in recruitment of epigenetic modulators and transcription factors to the promoter region. These findings indicate that overexpression of stemness gene Nanog in NCCIT cells is associated with maintaining stem cell-like phenotype and suggest that targeting Nanog might be an approach for improved therapy of poorly differentiated tumors. [Cancer Res 2009;69(14):5716–25]

Introduction

Phenotypic and functional similarities between tumor cells and normal stem cells have generated great interest in the possible links between these classes of cells (1). Both tumor cells and normal stem cells have extensive proliferative potential and the ability to give rise to new tissues, although normal stem cells do so in a highly regulated manner, whereas tumor cells self-renew in a poorly regulated manner and differentiate abnormally. Based on these similarities, it is hypothesized that tumors might arise from normal stem or progenitor cells (2). However, it is still possible that tumor cells can acquire stem cell-like characteristics through progressive dedifferentiation during their development. In addition, recent studies indicate that only a small subpopulation of tumor cells are clonogenic and have the exclusive ability to regenerate a tumor mass (3). These clonogenic cells have been found in a wide array of highly undifferentiated tumors including blood, brain, breast, and colon cancers with the capability of both self-renewal and at least partial differentiation, similar to that of normal stem cells (4). These characteristics of the cells have led to their designation as cancer stem cells or tumor-initiating cells with stem cell-like properties. Although some regulators of the stem cell function have been shown to be implicated in tumorigenesis, a detailed characterization of the activity of stem cell-associated regulatory networks in tumors is still lacking.

The core stemness transcription factors, Oct4, Sox2, and Nanog, which are expressed in embryonic stem cells and embryonic carcinoma cells, are thought to be central to the transcriptional regulatory network that specifies the identity of both cell types (5, 6). Oct4, a member of POU class of homeodomain proteins, is normally found in the inner cell mass of the blastocyst and is required to maintain the pluripotency of the inner cell mass cells (7). During differentiation, its expression is reduced through mechanisms involving epigenetic modification. Mice lacking Oct4 exhibit early embryonic lethality due to a failure to form the inner cell mass, indicating the critical role of Oct4 in controlling self-renewal of embryonic stem cells. In contrast, overexpression of Oct4 is directly linked to the tumorigenic potential of embryonic stem cells (8). Nanog, another transcription factor, also plays a critical role in regulating cell fate of the pluripotent inner cell mass during embryonic development (9). In the absence of Nanog, mouse embryonic stem cells differentiate into visceral/parietal endoderm, whereas overexpression of Nanog can override the requirement of leukemia inhibitory factor for self-renewal of embryonic stem cells (10). Similarly, Nanog overexpression in human embryonic stem cells enables maintenance of these cells in the pluripotent state for a prolonged period, and knockdown of Nanog promotes differentiation, indicating a role of Nanog in self-renewal of human embryonic stem cells (11). Additionally, forced expression of Nanog in hematopoietic stem cells followed by a transplantation of these cells leads to development of a lymphoproliferative disorder as well as γδ T-cell malignancy (12). Interestingly, Nanog and Oct4 are overexpressed in oral cancer stem-like cells enriched through sphere formation by cultivating oral squamous cell carcinoma cells (OSCC) from established OSCC cell lines or primary culture of OSCC patients (13). Moreover, OSCC patients positive to the expression of Nanog, Oct4, and CD133 have been associated with the worst survival prognosis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Recently, analysis of the expression patterns of gene sets associated with the embryonic stem cell identity in various human tumor types has identified a potential link between genes associated with embryonic stem cell identity and the histopathologic traits of tumors (14). Stemness genes such as Nanog, Oct4, Sox2, and c-Myc are normally enriched in embryonic stem cells, and activation targets of these genes have been shown to be overexpressed in histologically poorly differentiated tumors, whereas these tumors repress preferentially polycomb-related genes. This embryonic stem cell signature has been found in several poorly differentiated cancers and often associated with a poor clinical outcome. These observations suggest that stem cell-like characteristics shown by many poorly differentiated tumors might be attributed to abnormal activation of these genes.

Small molecules could offer several advantages that include the ability for a temporal, tunable, and modular control of specific proteins involved in various cellular processes without abnormal genetic modification (15). Cell-based phenotypic assay and pathway screening of synthetic small molecules and natural products have historically provided useful chemical tools to modulate and/or study complex cellular processes; thereby, some small molecules such as dexamethasone and all-*trans* retinoic acid have been identified as agents inducing stem cell differentiation. Moreover, histone deacetylase inhibitors (HDACI) and DNA demethylating agents, which target abnormal epigenetic modifications, induce differentiation of various transformed cells. However, the effects of these small molecules are not fully evaluated in embryonic stem cells or cancer stem cells. Therefore, the investigation of the potential of embryonic stem cell signature as molecular target for treatment of aggressive human tumors will provide a valuable rationale to develop an effective strategy for future cancer therapy.

In this study, we report the first evidence that HDACI apicidin is capable to down-regulate Nanog expression in poorly differentiated human embryonic carcinoma cells, which is mediated by a coordinated change in recruitment of epigenetic modulators (DNMT3B, CBP, and EZH2) and transcription factors (SP1, GCNF, Oct4, Nanog, and Sox2) to its promoter region. This down-regulation of Nanog expression leads to decrease of other stemness gene expression such as Oct4, Sox2, and their target genes and the induction of differentiation markers for all three germ layers including endoderm, mesoderm, and ectoderm. Furthermore, sensitivity to apicidin in suppression of Nanog expression is higher in embryonic carcinoma cells than in embryonic stem cells. This suggests that targeting the stemness gene Nanog may provide a novel strategy for therapy of poorly differentiated tumors associated with the embryonic stem cell signature.

Materials and Methods

Cell culture. NCCIT cells (American Type Culture Collection) were cultured in RPMI 1640 (Life Technologies), supplemented with 10% fetal bovine serum (Hyclone Laboratories) and 1% penicillin/streptomycin (Life Technologies).

Reagents and antibodies. Apicidin [cyclo(*N-O*-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)] was prepared from *Fusarium* sp. strain KCTC 16677 according to the method described (16). Commercial primary antibodies' information is described in Supplementary Information.

RNA extraction and reverse transcription-PCR. Total RNA was extracted using easy-Blue reagent (iNtRON Biotechnology). PCRs were run by using the gene-specific primers (Table S1). Detailed method and gene specific primers are described in Supplementary Information.

Western blot analysis. Cell lysates were boiled in Laemmli sample buffer for 3 min, and 30 μg of each protein were subjected to SDS-PAGE. Detailed method is described in Supplementary Information.

Gene expression profiling. Illumina's HumanRef-8 v2 BeadChips were used to generate expression profiles. All expression data are available at National Center for Biotechnology Information Gene Expression Omnibus under series no. GSE13177. The individual expression arrays are listed as GSM333027 to GSM333030. Detailed gene expression profiling method is described in Supplementary Information.

DNA methylation bisufite sequencing analysis. DNA was purified by using the G-DEX genomic DNA miniprep kit (iNtRON Biotechnology). Purified genomic DNA (1 μg) was treated with sodium bisulfite solution. Detailed method is described in Supplementary Information.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assays were done using the Acetyl Histone H3 Immunoprecipitation Assay Kit (Upstate Biotechnology) according to the manufacturer's instructions. Detailed chromatin immunoprecipitation method is described in Supplementary Information.

Cell proliferation, cell cycle, and apoptosis analysis assays. Cell proliferation assay was carried out with cell counting and Bromodeoxyuridine Colorimetric Cell Proliferation Kit (Roche Molecular Biochemicals). Cell cycle assays were done using Cycletest Plus DNA Reagent Kit (BD Biosciences) according to the manufacturer's instructions. The detail analyzing method is described in Supplementary Information.

Soft-agar colony-forming assay. NCCIT cells (1.0×10^5) were seeded in 0.4% agar and incubated at 37°C for 21 days. Colonies were visualized by 0.005% crystal violet staining and counted.

Small interfering RNA transfection. Cells were transfected with scrambled or target gene-specific small interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen). siRNAs specifically targeting Nanog or SP1 were purchased from Dharmacon and/or Invitrogen.

Results

Differential expression of stemness genes in various human cell lines. Poorly differentiated aggressive human tumors have been shown to possess embryonic stem cell signature, which is associated with poor prognosis (14). To examine the involvement of the key regulators of embryonic stem cell signature in tumorigenesis, we have analyzed the expression level of the core stemness genes in various human cell lines including embryonic stem H9, embryonic carcinoma NCCIT, normal fibroblast BJ, cervical carcinoma HeLa, breast cancer MCF7, ductal carcinoma HCC1954, osteocarcinoma U2OS, and lung carcinoma H1299 cells. Whereas Sox2 expression is detected in all cell lines tested with different levels, Oct4 and Nanog are expressed only in both embryonic stem H9 and embryonic carcinoma NCCIT cells (Fig. 1*A*). However, the level of Nanog expression is much higher in NCCIT than H9 cells, although Oct4 is expressed with a comparable level in both cells, indicating the dysregulation of Nanog expression in NCCIT cells.

Selective down-regulation of Nanog expression by HDACI apicidin in embryonic carcinoma cells. To identify the molecules that can suppress the abnormal Nanog expression in NCCIT cells, these cells have been treated with small molecules, which have been shown previously to regulate cell proliferation or differentiation, such as all-*trans* retinoic acid, reversine, sonic hedgehog signaling inhibitor (cyclopamine), mammalian target of rapamycin inhibitor (rapamycin), clinical anticancer agents (cisplatin, 5-fluorouracil), demethylating agent (5-aza-cytidine), and HDACIs (apicidin, trichostatin A, and suberoylanilide hydroxamic acid; ref. 17). Among the molecules tested, HDACIs (trichostatin A, apicidin, and suberoylanilide hydroxamic acid) have the most significant effect on down-regulation of Nanog mRNA and protein in a dose-dependent manner (Supplementary Fig. S1).

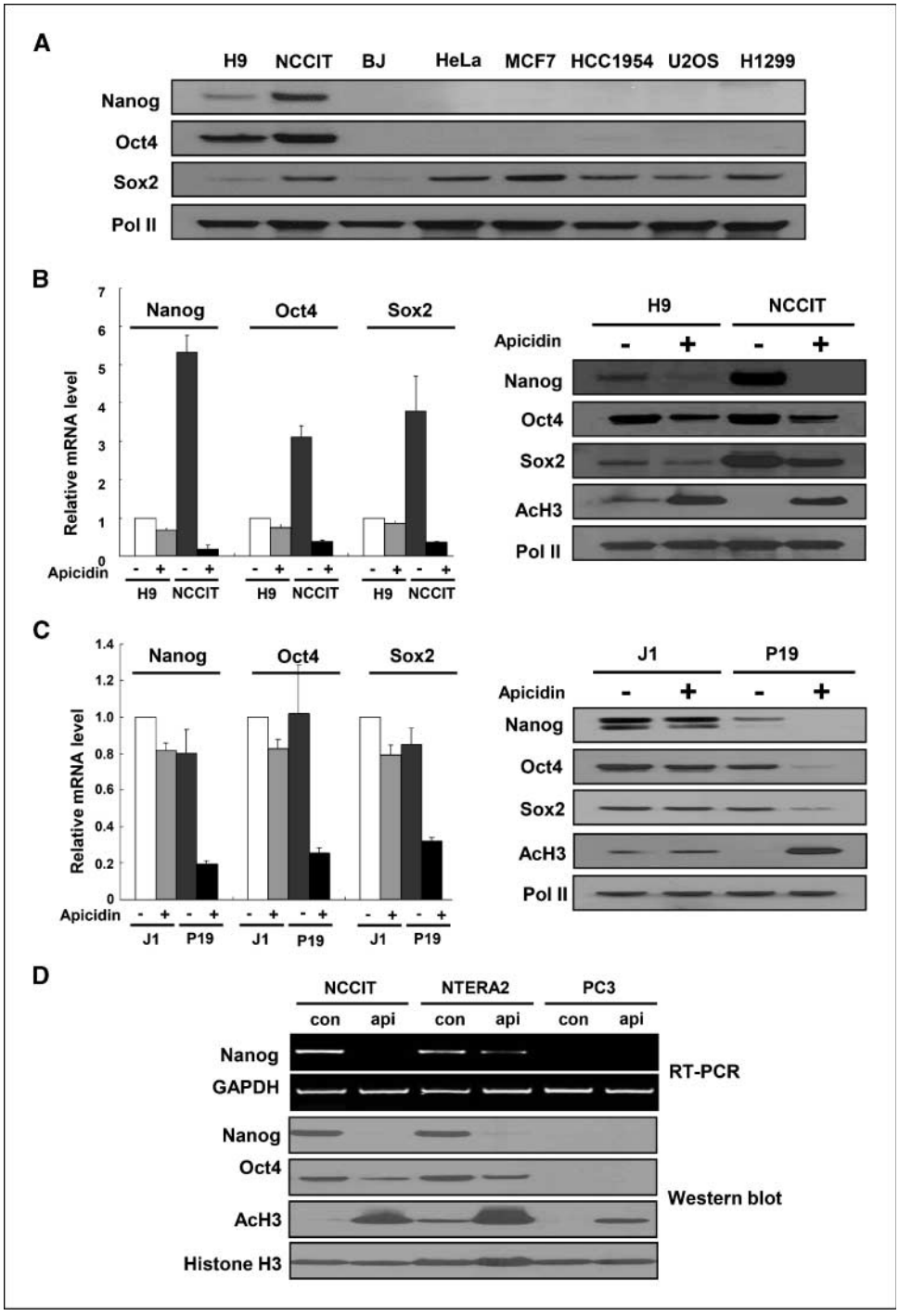

Figure 1. Differential expression of stemness genes in various human cell lines and apicidin-mediated down-regulation of stemness genes in embryonic carcinoma but not in embryonic stem cells. *A,* expression levels of stemness genes were determined by Western blot analysis in various human cell lines. *B,* human embryonic stem H9 and embryonic carcinoma NCCIT cells were exposed to 1 μmol/L apicidin. *C,* mouse embryonic stem J1 and embryonic carcinoma P19 cells were exposed to 0.5 μmol/L apicidin for 24 h. Cells were subjected to qPCR and Western blot analysis (*B* and *C*) for the expression of Nanog, Oct4, and Sox2. *D,* effects of apicidin on Nanog expression in other cancer cell lines were examined by RT-PCR and Western blot. Representative of three independent experiments. *con,* control; *api,* apicidin.

Among three HDACIs, we have further analyzed the effect of apicidin on Nanog expression in embryonic stem and embryonic carcinoma cells. Interestingly, apicidin treatment does not affect Nanog mRNA expression in normal human embryonic stem cells, whereas it causes a marked decrease in Nanog mRNA expression in malignant human embryonic carcinoma cells (Fig. 1*B*). The effect on Nanog protein expression is similar to that on Nanog mRNA expression, except a slight increase in sensitivity of human embryonic stem H9 cells to apicidin (Fig. 1*B*). Consistently, expression levels of Nanog mRNA and protein show no alteration in mouse embryonic stem J1 cells when treated with apicidin, indicating that embryonic carcinoma cells are more sensitive to apicidin treatment in inhibition of Nanog expression than embryonic stem cells (Fig. 1*C*). Interestingly, down-regulation of Nanog expression is accompanied by a decrease in Oct4 and Sox2 expression in both mouse and human embryonic carcinoma cells (Fig. 1). In addition, apicidin treatment leads to down-regulation of other stemness genes, such as FOXD3 and REX1 in NCCIT cells (data not shown), and induces a time- and dose-dependent decrease in Oct4, Nanog, and Sox2 expression in NCCIT cells (Supplementary Fig. S2). Among the stemness genes tested, Nanog expression is most sensitive to apicidin treatment, suggesting that

down-regulation of Oct4 and Sox2 expression might be mediated through down-regulation of Nanog. Consistent with these results, a selective knockdown of Nanog expression by two independent siRNA pools corresponding various regions of the Nanog transcript leads to a significant decrease in the expression levels of mRNAs as well as proteins of Oct4 and Sox2, whereas the control siRNA does not affect expression of these genes (Nanog, Oct4, and Sox2) and an irrelevant control gene (Supplementary Fig. S3). To further confirm the effects, we have analyzed changes of Nanog expression in two other poorly differentiated human cancer cell lines including NTERA2 (embryonic carcinoma line) and PC3 (a prostate adenocarcinoma cell line). Similarly, apicidin markedly decreases both Nanog mRNA and protein levels in NTERA2 cells, which express Nanog at high levels (Fig. 1*D*). However, the Nanog mRNA and protein in PC3 are not detected (Fig. 1*D*). These results indicate that apicidin down-regulates Nanog expression in Nanog-positive cancer cells.

Depletion of embryonic stem cell-like signature in NCCIT cells by apicidin. To evaluate the full potential of HDACI apicidin on transcriptional alterations in NCCIT cells, global gene expression profiles were determined by an Illumina HumanRef-8 v2 Expression BeadChips and compared between control and apicidin-treated NCCIT cells. Two thousand twenty-five genes of a total of 23,920 arrayed genes are affected by apicidin treatment with >2-fold significant changes relative to control NCCIT cells ($P < 0.05$; dataset S1). Among these genes, 1,030 genes are classified into the 13 gene sets associated with the human embryonic stem cell identity, which are classified previously by Ben-Porath and colleagues (ref. 14; dataset S2). The various embryonic stem cell-expressed and Nanog, Oct4, and Sox2 target gene sets are preferentially overexpressed in NCCIT cells, whereas the polycomb target gene sets are underexpressed (Fig. 2*A*). The apicidin treatment reverses enrichment patterns of all gene sets, except Myc targets 2 (Fig. 2*A*; Supplementary Fig. S4). Furthermore, the gene sets for Nanog, Oct4, and Sox2 transcription factors, embryonic stem cell expressions, Nanog, Oct4, and Sox2, and Nanog targets are markedly affected by apicidin (Fig. 2*A*). These findings indicate that the embryonic stem cell-like signature in NCCIT cells, if not all, could be depleted by HDACI apicidin.

Induction of differentiation and apoptosis of NCCIT cells by apicidin. To examine the effect of apicidin on induction of differentiation of NCCIT cells, we have analyzed the expression of various lineage-specific markers. The quantitative PCR (qPCR) analysis of mRNA expression reveals that apicidin induces the expression of the trophectoderm differentiation markers (CDX2 and CG), endoderm markers (GATA4, GATA6, AFP, FOXA2, SOX17, and CK18), mesoderm markers (BRACHYURY, MSX1, NKX2.5, NKX3.1, BMP4, HAND1, and MEF2C), and ectoderm markers (MAP2, PAX6, HES4, and OLIG2; refs. 5, 18), ranging from ~5- to 10-fold, compared with control (Fig. 2*B*), indicating the ability of apicidin to enhance the differentiation potential of NCCIT cells to the various lineages. To examine whether apicidin also affects cell death of NCCIT cells, the effect of apicidin on apoptosis of NCCIT cells has been analyzed by Annexin V staining using flow cytometry. Apicidin treatment leads to a significant increase in the number of Annexin V-positive cells (35.40%) compared with control (1.60%; Fig. 2*C*). The similar effect on apoptosis by apicidin treatment is also observed in NTERA2 and P19 cells (Supplementary Fig. S5). To further examine the effect of apicidin on anchorage-independent growth, we performed soft-agar colony-forming assay, which is considered the most stringent assay for detecting malignant transformation of cells. Apicidin treatment dramatically abrogates colony formation of NCCIT cells in soft agar (Fig. 2*D*). To further prove the role of Nanog in this model, we performed a rescue experiment by overexpressing Nanog. Overexpression of Nanog antagonizes the effects of apicidin on cell cycle arrest and differentiation (Supplementary Fig. S6).

Induction of antiproliferation and differentiation of NCCIT cells by knockdown of Nanog. To further investigate the relationship between the biological effects and the down-regulation of Nanog by apicidin, we have selectively inhibited Nanog expression using a siRNA targeting Nanog (Fig. 3*A*). The Nanog-depleted NCCIT cells show a prolonged doubling time as well as a significant reduction in bromodeoxyuridine incorporation, compared with the control cells, from 31% to 19% (Fig. 3*B* and *C*). To confirm the role of Nanog in cell growth, we rescued Nanog expression by replating cells without further transfection of siRNA. Suppression of cell growth by Nanog knockdown is recovered similar to that of control cells (Supplementary Fig. S3). In addition, NCCIT cells transfected with Nanog siRNAs form 62.3% less colonies than the control transfected cells (Supplementary Fig. S3). The analysis of lineage-specific markers has revealed that the effect of knockdown of Nanog on differentiation is similar to that observed by apicidin treatment (Fig. 3*D*). However, the Nanog knockdown has no significant effect on apoptosis (Supplementary Fig. S3), whereas the proliferative potential is markedly impaired. In addition, down-regulation of Nanog expression by apicidin treatment appears not to be attributed to apoptosis, because a low concentration of apicidin (0.1 μmol/L), which does not induce apoptosis of NCCIT cells, suppresses the level of Nanog expression. These results suggest that the effect of apicidin on induction of differentiation and antitumorigenicity might be mediated by down-regulation of Nanog but not the effect of apicidin on apoptosis.

Transcriptional inhibition of Nanog by apicidin. Apicidin has been shown to induce cell cycle arrest at G_0-G_1 in HeLa cells (19). To elucidate the relationship between apicidin-induced cell cycle arrest and down-regulation of Nanog, we have analyzed the effect of apicidin on cell cycle in NCCIT cells. In contrast to G_0-G_1 arrest in HeLa cells, apicidin induces G_2-M cell cycle arrest in NCCIT cells (Supplementary Fig. S5). To examine the possibility that the cell cycle arrest at G_2-M may regulate down-regulation of Nanog, NCCIT cells were arrested at G_2-M by treatment with a pharmacologic inhibitor nocodazole. As expected, the nocodazole treatment leads to cell cycle arrest at G_2-M, as seen by an increase in the number of cells at G_2-M up to 61% from 30% of control cells, comparable with that induced by apicidin (Supplementary Fig. S5). However, the G_2-M arrest by nocodazole fails to down-regulate Nanog expression, indicating that apicidin-induced down-regulation of Nanog expression might be independent of the apicidin-induced G_2-M arrest (Fig. 4*A*). In contrast to HDACI, DNA methylation inhibitor 5-azacytidine fails to induce neither cell cycle arrest nor down-regulation of Nanog (Fig. 4*A*; Supplementary Fig. S5).

To further characterize the mechanisms by which apicidin induces down-regulation of Nanog, we examined the recruitment of RNA polymerase II to the regulatory elements of the Nanog gene. The chromatin immunoprecipitation analysis using anti-polymerase II (8WG16) antibody shows that apicidin significantly suppresses the transcriptional initiation, resulting in a decrease in the transcriptional elongation as seen by a 65% and 68% decrease in recruitment of polymerase II into the transcription start site and coding region of the gene, respectively (Fig. 4*B* and *C*). These results suggest that apicidin might induce down-regulation of Nanog expression mainly via inhibition of transcription.

However, down-regulation of Nanog expression by apicidin appears to be attributed marginally to destabilization of Nanog protein, because the decrease of in Nanog protein level by apicidin is only slightly inhibited in the presence of a proteasome inhibitor MG132 (Supplementary Fig. S7).

Induction of repressive epigenetic modifications in the promoter of Nanog by apicidin. To examine whether down-regulation of Nanog by apicidin is associated with alterations of epigenetic modification on the promoter, we have first analyzed the status of DNA methylation, a repressive epigenetic marker. Bisulfite mapping reveals that methylation of four CpG sites located ~300 bp upstream of transcription start site (20) is affected by apicidin treatment. Unmethylated four CpG sites in the region undergo methylation following apicidin treatment (>30%; Fig. 4*D*). We have then performed chromatin immunoprecipitation analysis to test a possible involvement of histone modifications in transcriptional suppression of Nanog by apicidin. Apicidin treatment leads to the significant hypoacetylation of histone H3 and H4 on the region from transcription start site up to ~1,000 bp upstream harboring putative binding sites for transcription factors such as p53, SP1, Oct4, and Sox2. In addition, active marker K4 trimethylated H3 on nucleosomes associated with the promoter

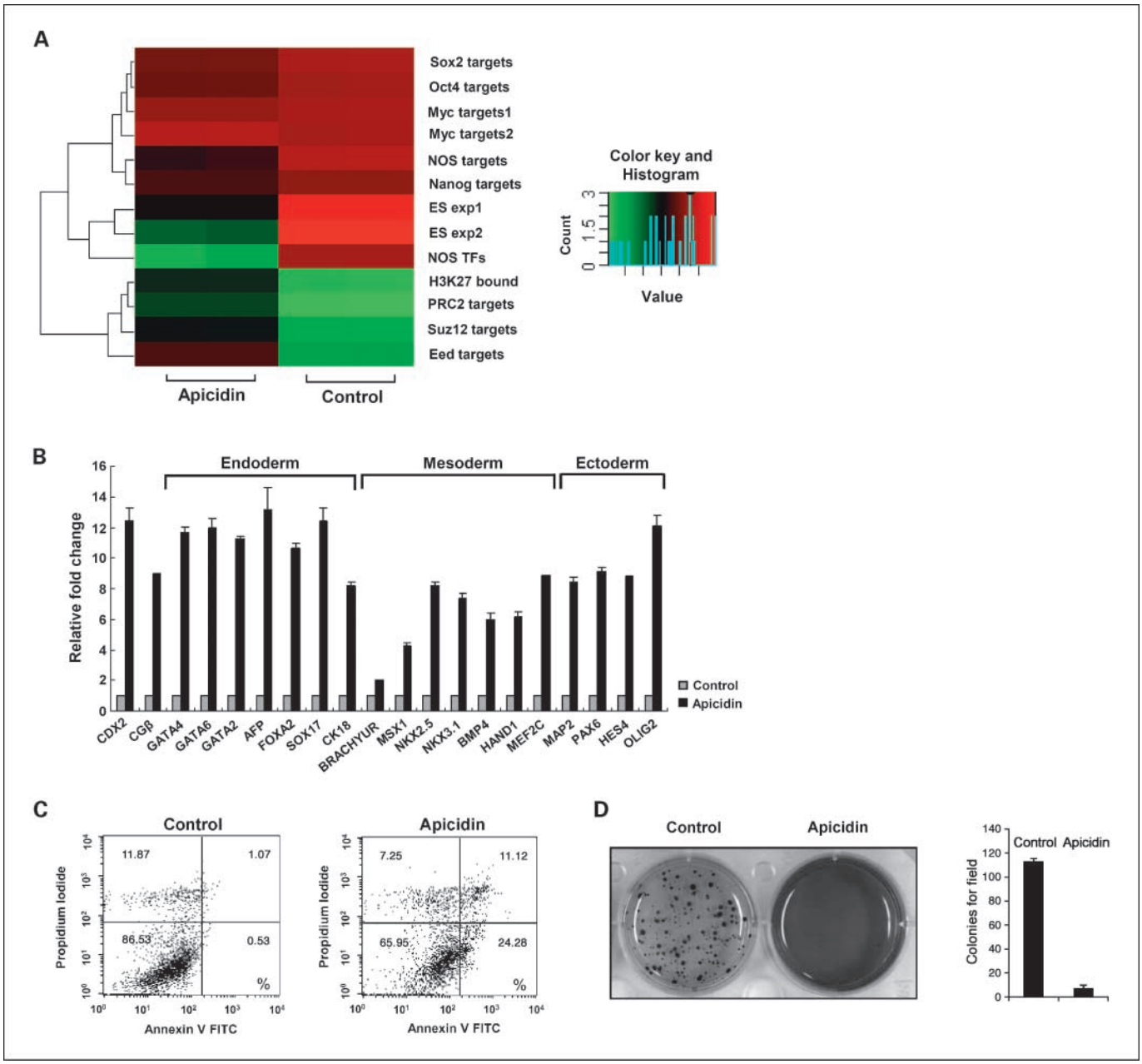

Figure 2. Apicidin selectively depletes embryonic stem cell-like signature and induces differentiation and apoptosis in NCCIT cells. *A,* cluster of gene sets associated human embryonic stem cell identity. Among the >2-fold cell significantly changed genes by apicidin (1 μmol/L, 24 h), 1,030 genes were matched with previously generated embryonic stem cell identity gene sets (datasets S1 and S2). *B,* effect of apicidin on differentiation in NCCIT cells. Expression levels of lineage-specific markers were determined by qPCR. *C,* extent of apoptosis of NCCIT cells in the absence or presence of apicidin (1 μmol/L, 24 h) was determined by flow cytometric analysis. Representative of three independent experiments. *D,* soft-agar colony-forming assay with/without apicidin treatment. Colonies were visualized by crystal violet staining and counted.

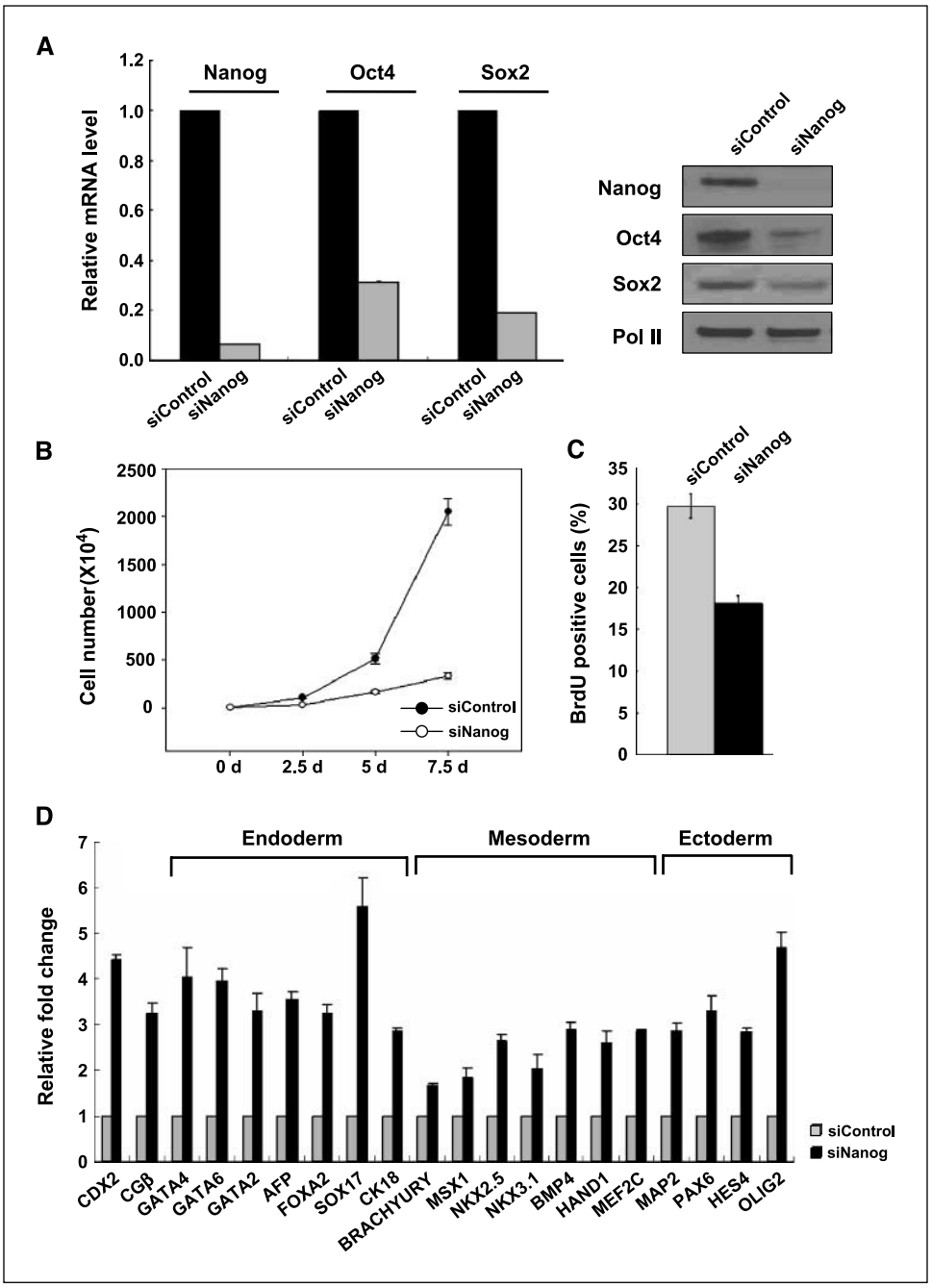

Figure 3. Nanog knockdown induces the antiproliferation and differentiation of NCCIT cells. Transfection of NCCIT cells with Nanog targeted (20 nmol/L) and control siRNA was carried out using Lipofectamine 2000. At 48 h post-transfection, the expression of target A, internal genes, and lineage-specific markers was analyzed by qPCR (*D*) and/or Western blot (*A*). *B* and *C,* at 48 h post-transfection, the proliferation is determined by cell counting for the indicated times and bromodeoxyuridine staining. *BrdUrd,* bromodeoxyuridine.

region of the Nanog gene is depleted following apicidin treatment, whereas the repressive marker K27 trimethylation of H3 is enriched on these sites. Consistent with the results, apicidin induces the recruitment of DNMT3B and EZH2 into the promoter while decreasing the recruitment of CBP (Fig. 4*E*). Chromatin immunoprecipitation data have further been confirmed by qPCR (Supplementary Fig. S8). Taken together, the results indicate that the transcriptional repression of Nanog by apicidin might be attributed to repressive epigenetic modifications (21) through alteration of the recruitment of epigenetic enzymes on the promoter.

Altered recruitments of transcription factors into the Nanog promoter by apicidin. Nanog gene is regulated by a complex interplay of both positive and negative *cis*-regulatory elements that are distributed over a large area of 5′ flanking region of the Nanog gene (22). Thus, we have analyzed the recruitment of negative/positive regulators into the promoter containing putative binding sites for transcription factors. On apicidin treatment, the Nanog, Oct4, Sox2, and SP1 are depleted at those sites (Fig. 5*A*). The decrease of the recruitment of Oct4 and Sox2 seems to be due to decreased protein levels of these genes as well as reduction of the binding affinity of these proteins to Nanog promoter, because apicidin (0.1 μmol/L), which suppresses the expression of Nanog without altering expression of Oct4 and Sox2, is able to block the recruitment of Oct4 and Sox2 to the Nanog promoter (Supplementary Fig. S9). In antiparallel, GCNF, a negative regulator (23), is

recruited into the region (b) (Fig. 5*A*). These data from chromatin immunoprecipitation analysis have been confirmed by the qPCR analysis (Supplementary Fig. S8). To further study the function of SP1 in apicidin-mediated down-regulation of Nanog expression, we have examined the effect of inhibitor of SP1 by the treatment of mithramycin, inhibitor of SP1 binding (24), or knockdown of SP1 by a SP1-specific siRNA on the Nanog expression. SP1 suppression by either mithramycin or siRNA ablates the expression of Nanog mRNA and protein (Fig. 5*B* and *C*).

Discussion

This study provides evidence that core stemness transcription factors Nanog, Oct4, and Sox2 could be targeted by HDACI for future therapy of poorly differentiated human tumors associated with embryonic stem cell signature. Treatment of embryonic carcinoma NCCIT cells with HDACI apicidin suppresses the expression of the stemness genes, Nanog, Sox2, and Oct4, of which Nanog is most sensitive to apicidin. The selective down-regulation of Nanog by siRNAs leads to a concomitant decrease in the levels of Oct4 and Sox2. Down-regulation of Nanog expression by apicidin is associated with transcriptional inhibition through induction of repressive epigenetic modifications in the promoter including DNA methylation, hypoacetylation of H3/H4, enrichment of K27 trimethylation, and depletion of K4 trimethylation of H3. Induction of the repressive epigenetic modification correlates well with coordinated changes of recruitment of epigenetic and transcriptional regulators. Furthermore,

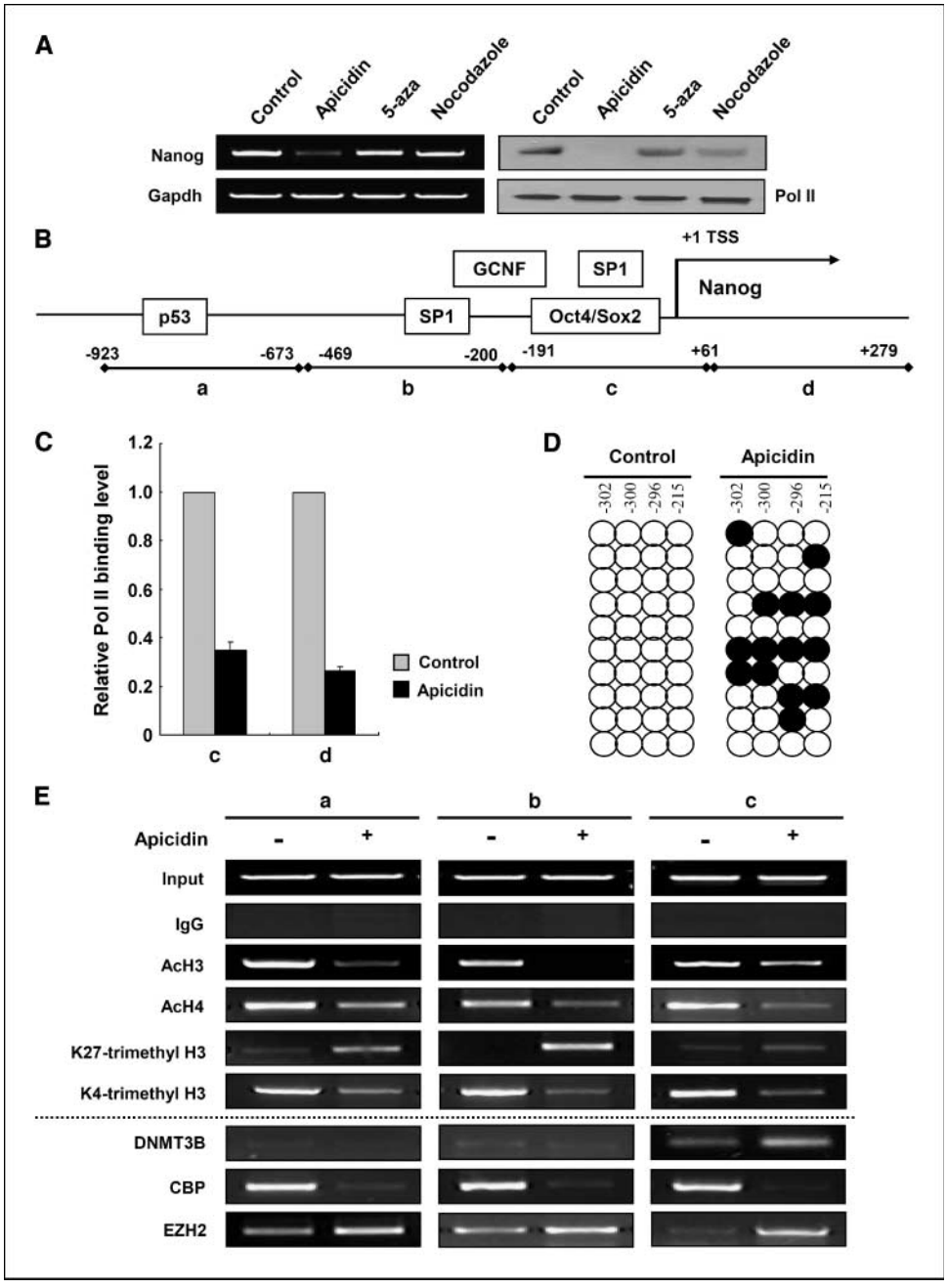

Figure 4. Apicidin inhibits Nanog transcription and induces repressive epigenetic modifications in the promoter of Nanog. *A,* NCCIT cells were incubated with apicidin (1 μmol/L, 24 h), 5-aza-cytidine (*5-aza*; 15 μmol/L, 24 h), or nocodazole (50 ng/mL, 16 h). mRNA and protein levels of Nanog were determined by RT-PCR and Western blot analysis, respectively, and normalized to GAPDH and polymerase II (*pol II*) loading controls. *B,* schematic diagram of the Nanog promoter, transcription initiation, and coding regions (*a-d*). Boxes mean putative transcription factor binding sites. *C* and *D,* chromatin was immunoprecipitated with anti-RNA polymerase II 8WG16 (*Pol II*) antibodies and DNA methylation profile of Nanog promoter was determined by bisulfide sequencing with or without apicidin (1 μmol/L, 24 h) in NCCIT cells. Polymerase II bindings to the transcription initiation and coding region of Nanog were analyzed by qPCR. CpG dinuceotide locations are indicated relative to transcription start site (*TSS*). Ten clones were sequenced and are shown for each site. *Open circles,* unmethylated cytosines; *closed circles,* methylated cytosines. *E,* changes of epigenetic modifications and changes in the recruitment of DNMT3B, CBP, and EZH2 into the promoter regions of Nanog in NCCIT cells were determined by chromatin immunoprecipitation analysis using the antibodies against acetylated or methylated histones and epigenetic modifying enzymes as described in Materials and Methods. Representative of three independent experiments with similar results.

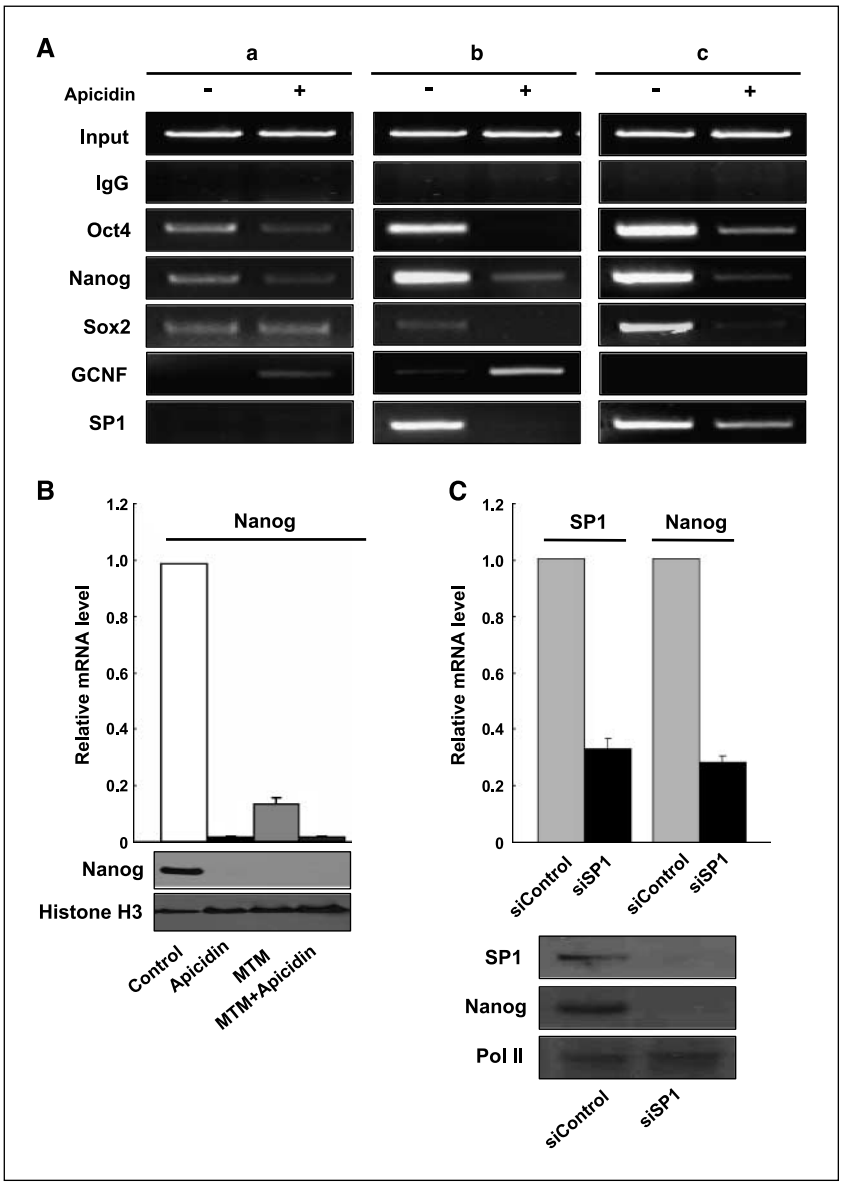

Figure 5. Effect of apicidin on the recruitment of transcription factors into the Nanog promoter. *A,* changes in the recruitment of Oct4, Nanog, Sox2, GCNF, and SP1 into the promoter regions of Nanog following apicidin treatment (1 μmol/L, 24 h) were determined by chromatin immunoprecipitation analysis in NCCIT cells. *B,* after pretreatment with 200 nmol/L mithramycin (*MTM*) for 1 h, NCCIT cells were incubated with 1 μmol/L apicidin for an additional 24 h. The level of Nanog was determined by qPCR and Western blot. *C,* transfection of NCCIT cells with SP1 targeted and control siRNA was carried out using Lipofectamine 2000. At 48 h post-transfection, the expression of SP1 and Nanog was analyzed by qPCR and Western blot. Representative of three independent experiments with similar results.

suppression of Nanog either by apicidin or siRNA enhances the potentials for antiproliferation, differentiation, and antitumorigenicity of NCCIT cells. These results indicate that HDACI can be efficiently used to treat aggressive human tumors with embryonic stem cell signature.

Embryonic carcinoma cells provide a good model system of the stem cell concept of cancer, because they are the stem cells derived from a teratocarcinoma, and the malignant counterparts of embryonic stem cells, capable of self-renewal as well as differentiation into a wide range of cell types, similar to embryonic stem cells (25, 26). In addition, embryonic carcinoma cells show profiles of gene expression very similar to those of human embryonic stem cells (6). In particular, the core transcriptional regulatory circuitry consisting of Nanog, Oct4, and Sox2 is retained active in embryonic carcinoma cells, indicating that the core stemness genes might be good molecular targets to treat poorly differentiated human tumors (1, 8, 13, 14, 27). Among them, Nanog gene has also been shown to be located on human chromosome 12p13, a region frequently duplicated on human tumors of germ cell origin including embryonic carcinoma cells and human embryonic stem cells cultured for a long period (6, 28). Indeed, Nanog is overexpressed much higher than other stemness genes, Oct4 and Sox2, and most sensitive to HDACI in NCCIT cells (Fig. 1). Furthermore, HDACI suppression of Nanog expression precedes decrease of Oct4 and Sox2, and the specific knockdown of Nanog by siRNAs induces the down-regulation of Oct4 and Sox2 expression (Figs. 1 and 3; Supplementary Information). These results suggest that Nanog abnormally overexpressed in NCCIT cells may act as a master transcription regulator that entrains the hierarchy of gene expression controlling pluripotency. In contrast to our findings, it has been reported previously that Nanog knockdown in NCCIT cells had no effect on expression levels of Oct4 and Sox2 (29). This discrepancy might be due in part to the incomplete knockdown of Nanog in the previous study, because complete knockdown of Nanog by transfecting a combination of the siRNA pool from previous

study and a second siRNA pool corresponding to different regions of the Nanog transcript leads to concomitant suppression of Oct4 and Sox2 (Fig. 3*A*; Supplementary Fig. S3). Importantly, apicidin-induced suppression of the key regulators (Nanog, Oct4, and Sox2) is accompanied by reversion of the enrichment patterns of 13 gene sets associated with embryonic stem cell identity and by induction of cell cycle arrest at G_2-M and expression of differentiation markers for all three germ layers, suggesting that the depletion of embryonic stem cell signature in NCCIT cells might be sufficient for inhibition of proliferation, leading to differentiation.

The epigenetic regulation of stemness genes is critical for maintaining self-renewal and pluripotency of stem cells (30, 31). Thus, abnormal expression of Nanog in NCCIT cells might be attributed to dysregulation of epigenetic control in addition to genetic aberration. In fact, the Nanog promoter is marked by active epigenetic modifications, which is reversed by apicidin treatment; apicidin induces DNA methylation of four CpG sites in association with repressive histone modifications, K27 trimethylation, depletion of K4 trimethylation of H3, and hypoacetylation of H3/H4 (Fig. 4). In parallel, CBP is dissociated from the promoter, whereas both EZH2 and DNMT3B are recruited to the region (c) proximal to the transcription start site (Fig. 4). EZH2 has been shown to control CpG methylation through a direct physical contact with DNMTs (32). Thus, the recruitment of EZH2 and DNMT3B to the promoter might be medicated by protein-protein interaction between EZH2 and DNMT3B, resulting in generation of pivotal repressive markers, methylation of four CpG sites, and K27 trimethylation. The levels and recruitment of other epigenetic modifiers such as HDACs, P/CAF, and DNMT1 into the promoter are minimally affected by apicidin (data not shown). Changes of chromatin structure by epigenetic modifications are associated with alteration of accessibility of transcription factors to the promoter (33). Alteration of epigenetic modifications on the Nanog promoter by apicidin is accompanied by decrease in the recruitment of positive transcription regulators (Nanog, Oct4, Sox2, and SP1) and increase in the recruitment of negative regulator GCNF (Fig. 5). The decrease of the recruitment of Nanog, Oct4, and Sox2 seems to be attributed to regulatory circuitry consisting of autoregulatory and feed-forward loops between them as shown previously (5). However, in addition to the regulatory circuitry, other unknown mechanism independent of Nanog appears to be involved in down-regulation of Oct4 and Sox2 by apicidin, because 0.1 μmol/L apicidin (Supplementary Fig. S2) can suppress only Nanog expression without alterations in Oct4 and Sox2 expression, whereas down-regulation of Nanog by Nanog siRNAs and 1 μmol/L apicidin (Figs. 1 and 3*A*) is accompanied with suppression of Oct4 and Sox2. Recent studies have shown that, on treatment of mouse embryonic stem cells with retinoic acid, induction of GCNF and p53 suppresses the expression of Nanog via direct binding to the promoter, resulting in differentiation (22, 23, 34). Consistent with these observations, we show that GCNF is recruited to the promoter region harboring putative binding sites for transcription factor GCNF by apicidin, which is accompanied by differentiation. In contrast, the recruitment of another negative regulator p53 is not detected (data not shown). This appears to be due to expression of truncated p53 by a frameshift mutation in NCCIT cells, because p53 recruitment to the Nanog promoter is detected in normal p53-expressing mouse embryonic carcinoma P19 cells (data not shown). Recently, mSin3A/HDAC complex has been shown to play a positive role in Nanog and Sox2 promoters in mouse embryonic carcinoma P19 cells (35). However, we could not detect significant changes in the recruitment of mSin3A in NCCIT cells by apicidin treatment, although we observed a positive role of mSin3A in Nanog promoter in P19 cells as reported previously (Supplementary Fig. S8). Forced expression of Oct4 and Sox2 in differentiated cells fails to rescue Nanog promoter activity, indicating that some other transcription factors may be required for induction of Nanog gene expression. Furthermore, SP1 binding sites have recently been mapped on the Nanog gene promoter in mouse embryonic carcinoma cells (36). In agreement with this notion, SP1 is enriched at the promoter containing GC-rich elements in NCCIT cells but depleted in the presence of apicidin. This is further evidenced by the observation that SP1 knockdown by either mithramycin or siRNA also ablates the expression of Nanog (Fig. 5). Because it has been shown that SP1 is able to form a coactivator complex with p300/CBP, the decrease of SP1 recruitment by apicidin might lead to concomitant dissociation of CBP from the promoter, thereby hypoacetylation of histone H3 and H4 (37). Overexpression of CBP causes a slight enhancement of Nanog expression in the absence of apicidin but does not restore down-regulation of Nanog by apicidin (Supplementary Fig. S8). These results indicate that a change of the SP1affinity to the promoter rather than the level of CBP protein is more crucial for down-regulation of Nanog by apicidin. However, it appears that SP1 alone is not sufficient, because Nanog is not detected in other cancer cells expressing SP1 (Fig. 1; Supplementary Fig. S1).

Understanding the embryonic stem cell signature in poorly differentiated human tumors will be helpful in eradicating tumors more efficiently. One promising approach is to target the core stemness transcription factors, Nanog, Oct4, and Sox2, which are well known for playing important roles in self-renewal and pluripotency of stem cells. Our study shows that Nanog abnormally overexpressed by epigenetic and transcriptional dysregulation may contribute to cancer characteristics of poorly differentiated aggressive tumors and provides insights into the mechanism of Nanog transcriptional regulation in embryonic carcinoma cells. Furthermore, the observation that HDACI is able to deplete Nanog with concomitant suppression of Oct4 and Sox2 suggests that development of specific drugs that target abnormal patterns of epigenetic modification may be not only an attractive strategy to modulate gene expression patterns, which confer unique properties in cancer cells defined as stemness, but also a useful pharmacologic tool for studying the fundamental biology of stem cells, which is now emerging as an important and exciting field.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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