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Original Articles

TET1 promotes 5hmC-dependent stemness, and inhibits a 5hmC-independent epithelial-mesenchymal transition, in cervical precancerous lesions

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ABSTRACT

DNA hypermethylation is a driving force in carcinogenesis. However, the role of active DNA hypomethylation in cancer remains largely unknown. This process, facilitated by ten-eleven translocation methylcytosine dioxygenase 1 (TET1), which oxidizes 5-methylcytosine (5 mC) to 5-hydroxymethylcytosine (5hmC), has never been studied in cervical cancer. Here, we found that TET1 and 5hmC correlative increases from normal cervix to Low-grade squamous intraepithelial lesion (LSIL), maximizing in High-grade squamous intraepithelial lesion (HSIL), and decreasing in invasive cancer. Full-length HPV-immortalized HSIL cells demonstrated higher TET1/5hmC levels, and stemness properties, compared to invasive cancer cells. *TET1* silencing promoted the epithelial-mesenchymal transition (EMT), to transform precancerous cells *in vivo*. TET1 increased 5hmC in the *ZEB1* and *VIM* promoters, surprisingly, silencing both genes. TET1 interaction with the histone modifiers, LSD1 and EZH2, on the *ZEB1* promoter, resulted in gene silencing, via loss of histone H3K4 trimethylation, and gain of histone H3K27 trimethylation. Taken together, TET1 promotes stemness properties, and inhibits EMT, in HSIL cells, through 5hmC-dependent and -independent mechanisms.

1. Introduction

Cervical cancer is the fourth-most common female cancer, and the seventh overall cause of cancer death, in the world. In 2015, there were an estimated 530,000 worldwide cases, and 270,000 deaths [1]. Over 90% of deaths from cervical cancer occur in developing countries. However, even in developed countries, 11,572 and 175,229 annual new cases occur in the U.S and Southeast Asia, respectively [2]. Although effective vaccines have now been in use for over a decade, most of the world's female population remains unvaccinated [3].

The causative agent of cervical cancer is the human papillomavirus (HPV) [4]. Most HPV infections are transient, leaving only a small number of cells persistently infected, insufficient for cervical epithelial transformation, which requires the accumulation of subsequent epigenetic aberrations [5]. Furthermore, such aberrations, such as histone and DNA methylation anomalies, are required for cervical cancer progression [6].

DNA hypermethylation of tumor suppressor genes is now well known to occur in cervical cancer [7], as HPV infection interferes with epigenomic homeostasis in the cervical epithelium [8]. Specifically, the HPV “early gene” E6 is believed to upregulate DNA methyltransferase 1 (DNMT1) [9], subsequently resulting in gain of DNA methylation, after replication. In addition, HPV E7 can bind to, and augment, DNMT1 enzymatic activity, effectively silencing the tumor suppressor *CDH1* (E-cadherin) [10]. Thus, DNA hypermethylation, as a cervical cancer early detection biomarker, adjunct to cytology or HPV testing, is under

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extensive investigation [11–13].

Contrary to the gain of methylation, the role of DNA demethylation, in oncogenesis, remains largely unknown. Loss of DNA methylation can be active or passive. Passive demethylation is replication-dependent, largely due to reduced DNMT activity and various DNA repair mechanisms, during DNA replication [14]. Active DNA demethylation, by contrast, is mediated by the ten-eleven translocation methylcytosine dioxygenase (TET) family, which modifies the methyl group by oxidizing 5-methylcytosine (5 mC) to 5-hydroxymethylcytosine (5hmC), which is subsequently removed by base excision repair [15]. To date, *TET1* is known as highly expressed in embryonic stem cells and neurons [16], and is also essential for phenotype reprogramming ("plasticity") in induced pluripotent stem cells [17].

Although downregulation of TET1, and loss of 5hmC, have been observed in a few cancers [18], and correlated with poor prognosis in colon and breast cancer [19,20], the role of these events in cancer in general (especially with regard to stemness), is limited. While it is known that most normal cells do not express TET1, or at very low levels [21], it remains elusive whether such lowly expressed TET1 can serve as a tumor suppressor, especially in early carcinogenesis. Consequently, herein, we hypothesized that TET1 might act as an early tumor suppressor in the cervix, using HPV-immortalized cervical carcinoma cells as a unique model.

2. Materials and methods

2.1. Clinical samples and immunohistochemistry (IHC)

Paraffin-embedded cervical tissues of patients were retrieved from the Department of Pathology, National Defense Medical Center, Taiwan. The tissue microarrays comprised histologically normal squamous epithelium (n = 22), low-grade squamous intraepithelial lesion (LSIL) (n = 16), high-grade squamous intraepithelial lesion (HSIL) (n = 21) and squamous cell carcinoma (SCC) (n = 65). All patients were diagnosed, treated, and had their tissues banked at the National Defense Medical Center, Taipei, Taiwan. The final diagnosis was made by tissue-proven pathology rather than cytology except for controls. Exclusion criteria included pregnancy, chronic or acute systemic viral infections (except HPV infection), a history of cervical neoplasia, skin or genital warts, an immunocompromised state, the presence of other cancers. or a history of surgery to the uterine cervix. The IHC procedure followed a standard protocol, using the indicating antibodies. All tissue microarray slides were examined and scored by pathologists, according to the summation of the percentage of area stained multiplied by the stain intensity. This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the Tri-Service General Hospital (Approval date: 12/27/2013; approval number: 2-1001-05-137). All of the patients signed informed consent forms before study.

2.2. Cells and cell culture

Five human cervical cell lines Z172, Z183, SiHa, HeLa, and CaSki (American Tissue Type Collection) were used in this study. Z172 and Z183 represent pre-cancerous cervical epithelia, while SiHa, HeLa, and CaSki are fully malignant. Of these, Z172, SiHa and CaSki are HPV-16 positive, Z183 and HeLa are HPV-18 positive. Z172 and Z183 were cultured in supplemented Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO®) containing 10% NuSerum IV (Collaborative Research). HeLa and SiHa were cultured in supplemented DMEM containing 10% fetal bovine serum (FBS). CaSki was cultured in Roswell Park Memorial Institute (RPMI) 1640 (GIBCO®) medium supplemented with 10% FBS. All culture medium containing 1.5 mg/ml sodium bicarbonate, 1 mg/ml HEPES. Cells cultured at 37 °C incubator with 5% CO_2. The TET1 shRNA LKO.1 vector was purchased from the National RNAi Core Facility (Academia Sinica, Taiwan, ROC), having the following target sequences: 5′- CCGGAGTTCAGTTACGATATCATGTCTCGAGACATTCG CGAGTAACTGAACTTTTTT -3′ (shScramble) and 5′-CCCAGAAGATTT AGAATTGAT-3′ (shTET1).

2.3. RNA isolation and reverse transcription

Cell pellets were homogenized in TRIzol reagent (Invitrogen/Life Technologies), according to the manufacturer's recommendations, and the concentration and purity of each RNA sample was assessed by measuring the ratio of absorbances at 260 nm and 280 nm (A_{260}: A_{280} ratio). Reverse transcription was performed using random hexamers with Transcriptor First Strand cDNA Synthesis kits (Roche Molecular Systems), according to the manufacturer's recommendations. The conditions of reverse transcription were incubation at 50 °C for 60 min, and 85 °C for 5 min.

2.4. Real-time quantitative PCR

For quantification of gene expression, using SYBR Green PCR Master Mix reagent kits (LightCycler®480 SYBR Green I Master, Thermo Fisher Scientific), according to the manufacturer's instructions. Q-PCR was performed on a LightCycler® 480 Instrument (Roche Molecular Systems), using the primers listed in Supplementary Table S1, including the housekeeping gene *GAPDH* as an internal control. PCR cycling was as follows: 95 °C for 10 min, prior to 45 cycles of 95 °C for 15 s, and 65 °C for 1 min. The final step, melting curve analysis, was used to determine the purity of the PCR product, ideally as a single peak. The relative expression level of a target gene was based on the levels of the housekeeping gene, in both normal or control samples, according to the $2^{-\triangle\triangle Ct}$ method. The primers used in this study are shown in Supplementary Table S1.

2.5. Chromatin immunoprecipitation (ChIP)

10^7 cells were fixed with formaldehyde, and ChIP performed using a Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Merck Millipore). The ChIPed DNA was then used for q-PCR to detect and amplify the putative promoter-binding region. Antibodies used for ChIP analysis were anti-TET1 (GTX627420, GeneTex), anti-5-Hydroxymethylcytosine (5-hmC) (39769, Active Motif), anti-LSD1 (A300-215A, Bethyl), anti-EZH2 (5246, Cell signaling), anti-H3K4me3 (17-614, Merck Millipore), and anti-H3K27me3 (17-622, Merck Millipore). The primers used in this study are shown in Supplementary Table S2.

2.6. Proliferation assay

3000 cells were seeded in 96-well plates, and on the indicated day, proliferation assay reagent was prepared, in 100 µl total medium, with 20 µl MTS reagent (C CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega) per well. Culture media was then removed, proliferation assay reagent was added at 120 µl/well, and the cells incubated at 37 °C for 1 h. The absorbance at 490 nm was recorded using a microplate reader.

2.7. Wound-healing assay

1.5×10^6 cells/well were seeded in 6-well plates, and cultured for 1 day to reach ~90% confluence as a monolayer The monolayers were then gently and slowly scratched with a new 1-ml pipette tip across the well, and the process then repeated by scratching a line perpendicular to the first line, creating a cross. The wells were then washed, and pictures then taken at time = 0 h. The cells were then grown for 24 h, and time-specific pictures taken to show migration. The differences in size of the areas ("wounds"), at the two time points, were determined by ImageJ software.

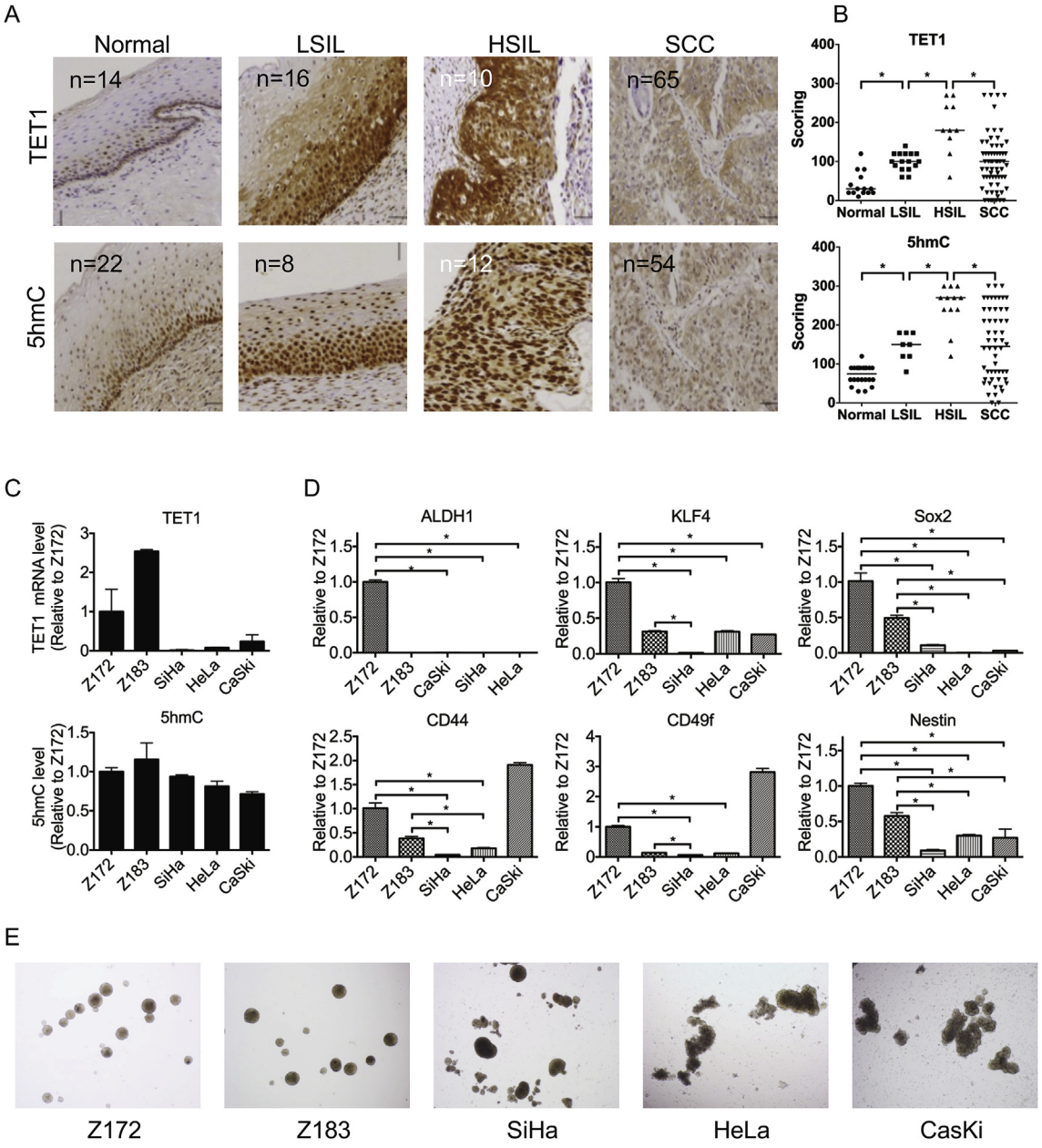

Fig. 1. TET1 expression correlates with stemness phenotypes in cervical precancerous cells. (A–B) The status of TET1 and 5hmC in samples across the full spectrum of cervical lesions. (C–D) Levels of TET1, 5hmC, and stem cell-related genes were higher in Z172 and Z183 precancerous cell lines, compared to the cancer cell lines, HeLa, SiHa, and CaSki. (E) Spheroid formation, a stemness phenotype, by Z172 and Z183 cells, was higher than in HeLa, SiHa, and CaSki invasive cancer cells.

2.8. Co-immunoprecipitation (co-IP)

Cell pellets were then extracted to isolate total nuclear protein, using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Concentrations of the nuclear protein samples were then determined by Pierce™ BCA Protein Assay Kits (Thermo Fisher Scientific). BSA was used as a standard, and measured by absorbance at 595 nm.

For immunoprecipitation (IP), 2.4-mg nuclear protein was used with a Pierce™ Classic Magnetic IP/Co-IP Kit (Thermo Fisher Scientific), with the above-mentioned antibodies. IP'ed protein was then solubilized in a sample buffer (Bio-Rad Laboratories), with 1% 2-mercaptoethanol, and heated at 95 °C for 5 min. After denaturation, proteins were separated in a 6% (v/v) sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel, and then transferred to polyvinylidine difluoride membranes (PVDF, Millipore).

Following membrane transfer, nonspecific binding sites on the membrane were blocked with 5% BSA in phosphate-buffered saline/Tween-20 (PBST), for 1 h, at RT, and then incubated with the indicated primary antibodies, at 4 °C, overnight. Next, the membranes were washed with PBST 3 times, incubated with a peroxide-conjugated secondary antibody for 1 h at room temperature, and an ECL Kit (Millipore) used to detect peroxidase activity, with readout on a Diagnostic Instruments imager (SPOT)/FLEX™, UK). The antibodies used for IP and western blot were anti-TET1 (GTX627420, GeneTex), anti-LSD1 (A300-215A, Bethyl), anti-EZH2 (5246, Cell signaling).

2.9. Genomic DNA extraction and dot blot

5 × 106 cells were prepared by DNeasy® Blood & Tissue Kit

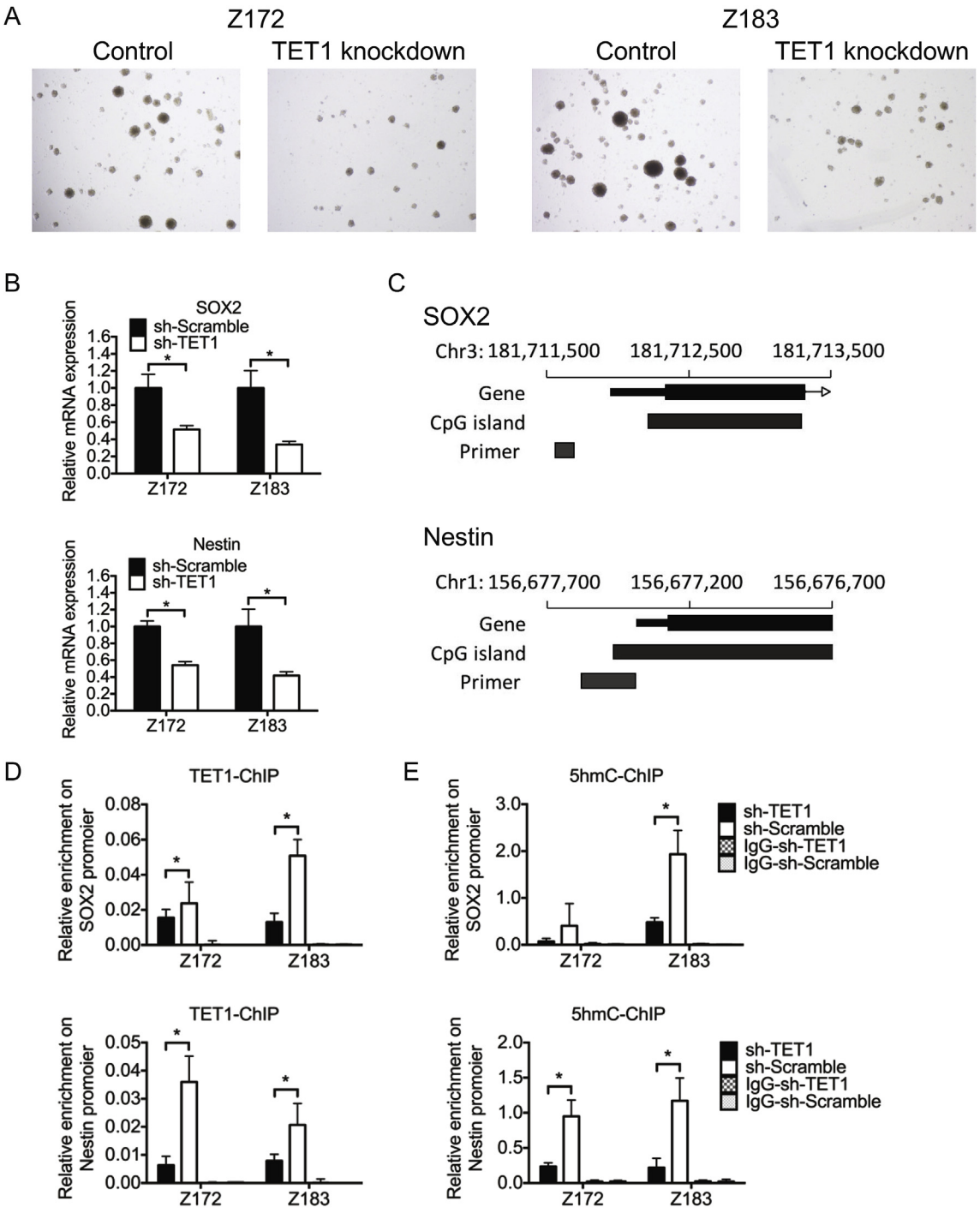

Fig. 2. *TET1* knockdown reduces stemness phenotypes in precancerous cells. (A) Spheroid formation and (B) stem cell-related genes expression, *SOX2* and *Nestin*, were reduced after *TET1* knockdown. (C) The location of ChIP-PCR primers in the promoters of *SOX2* and *Nestin*. (D) ChIP–qPCR showed that TET1 binding to the *SOX2* and *Nestin* promoters was reduced, after *TET1* knockdown, in precancerous cells. loss of TET1 correlated with loss of 5hmC, within the two gene promoters (E).

(QIAGEN) according to the manufacturer's recommendations. DNA (350 μg) was spotted onto a nitrocellulose membrane and air-dried. The completely dried membrane was baked for 30 min at 80 °C and then blocked with TBS containing 5% nonfat milk and 0.1% Triton X-100 for 1 h at room temperature. To control for spotting, blots were stained with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.2). Dots were quantified using ImageJ software (30). The antibody used in the dot blot analysis was anti-5-Hydroxymethylcytosine (5-hmC) (39769, Active Motif).

2.10. Xenograft model of TET1 KD in precancerous cells

Six-week-old female inbred NOD/SCID mice were obtained from BioLASCO Taiwan Co., Ltd. Mice were allowed to acclimate to animal housing for 7 days, before study. Using a 1-cc syringe and a 29-gauge needle, 5×10^6 *TET1*-knocked down, or scrambled control, precancerous cells were subcutaneously (s.c.) injected by into each murine flank. The protocol for this animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Defense Medical Center, Taipei, Taiwan. All animal procedures and

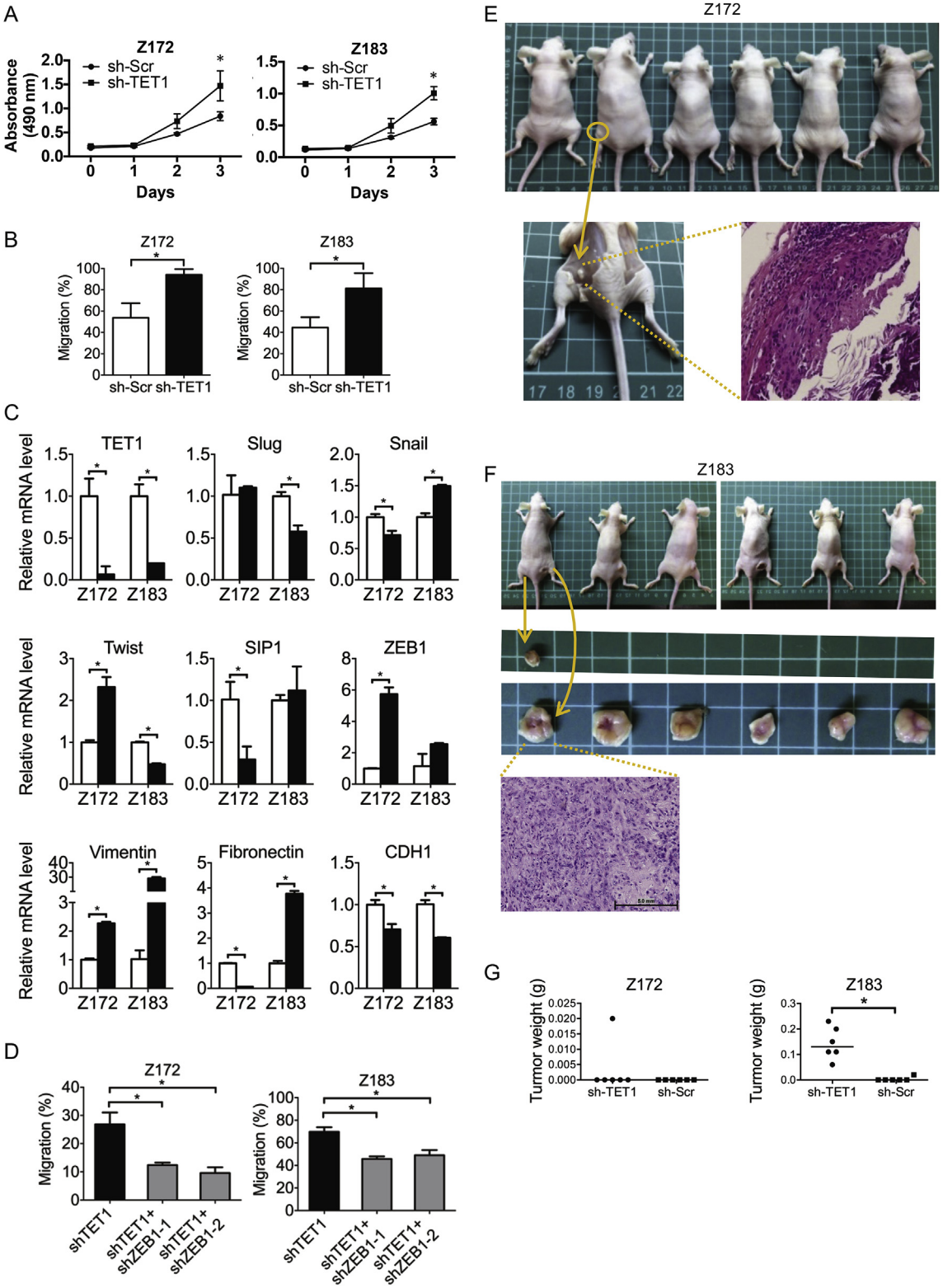

Fig. 3. *TET1* knockdown promotes proliferation and EMT, in precancerous cells, *in vitro* and *in vivo*. Both proliferation (A) and migration (B) increased after *TET1* knockdown. (C) *TET1* depletion upregulated various EMT transcription factors and markers. (sh-Scramble, black bar; sh-TET1, white bar) (D) The absence of *ZEB1* attenuated the migration ability of *TET1*-knocked down precancerous cells. (E–F) TET1 knockdown promotes tumor formation in precancerous cell lines, *in vivo*. (G) Tumor weights at the end of the *in vivo* experiment.

animal care were performed according to institutional animal research guidelines.

2.11. Statistical analysis

The Mann–Whitney *U* test, two-tailed, was used to compare data groups for cell proliferation, migration, and tumor formation. Standard deviations were used for error bars and various comparisons. All analyses were two-sided, and *p*-values < 0.05 were regarded as significant. $^{*}p < 0.05$. All statistical calculations were performed using the statistical package SPSS version 20.0 for Windows (IBM Corp.).

3. Results

3.1. TET1 overexpression in cervical precancerous lesions

To test our hypothesis that TET1 is upregulated in precancerous lesions, and downregulated in invasive cancers, we measured its expression across the full spectrum of cervical lesions, by immunohistochemistry (IHC). Those results showed that TET1 expression was rare in normal squamous epithelium, increased with severity of intraepithelial lesion (i.e., low-grade squamous intraepithelial lesion. LSIL), peaked in high-grade squamous intraepithelial lesion (HSIL) tissues, and was again suppressed in invasive squamous cell carcinoma (SCC) tissues. The median of TET1 level in normal squamous epithelium, LSIL, HSIL and SCC are 30, 100, 180 and 100, respectively. TET1 downregulation also correlated well with downregulation of 5hmC levels (The median of 5hmC level in normal squamous epithelium, LSIL, HSIL and SCC are 75, 150, 270 and 145, respectively.) (Fig. 1A and B).

To provide further evidence of TET1 expression, in precancerous lesions, we developed a model using two cervical precancerous cell lines, Z172 and Z183, representing cervical epithelial cells immortalized by full-length HPV16 and HPV18, respectively. Neither Z172 nor Z183 cells could form tumors *in vivo* [22], affirming their recapitulation of precancerous cervical cancer. The latter was also supported by Z172's and Z183's ability, as host cells, to regulate HPV viral genes (e.g., E2 and the long control region), in contrast to widely used precancerous cervical cell lines transformed by SV40 promoter-driven HPV E6/E7. Consequently, Z172 and Z183 cells better contextualized HPV-infected premalignant cervical epithelial cells, compared to traditionally immortalized cells. In that regard, and consistent with our IHC results, we found that both TET1 and 5hmC levels were upregulated in these two cervical precancerous cell lines, as compared to invasive cell lines (Fig. 1C). Together, these findings imply a role for TET1 in suppressing early carcinogenesis.

3.2. TET1 promotes stemness in precancerous cells

Since most published literature has reported the unique role of TET1 in embryonic stem cells and tissue progenitor cells, this spurred our interest in TET1's role in stemness of precancerous cells. Consequently, we assessed the presence of stemness immunotype markers, in addition to sphere formation, in Z172 and Z183 cells. Those results showed significantly higher expression of stem markers, albeit with Z172 and Z183 cells having different expression profiles (Fig. 1D). In particular, the pluripotency inducers *SOX2* and *NESTIN* were consistently highly expressed, in the two precancerous cell lines, in positive correlation with sphere formation ability, as compared to the fully cancerous cell lines (Fig. 1E). With regard to the latter, *TET1* knockdown reduced sphere formation, both in number and size (Fig. 2A and Supplementary Fig. S1), and decreased levels of TET1 and 5hmC in the *SOX2* and *NESTIN* promoters, in association with downregulation of those two genes (Fig. 2B–E). These results indicate that TET1 promotes stemness phenotypes in HPV-immortalized precancerous cells.

3.3. Loss of TET1 transforms HPV-immortalized precancerous cells

It is believed that epigenetic homeostasis prevents invasion of HSIL [23]. Moreover, the epithelial-mesenchymal transition (EMT) is well established to play a role in cancer invasion and metastasis [24]. Since TET1 was strongly expressed in HSIL, but not in invasive cancers, we hypothesized that TET1 may inhibit EMT of precancerous cells. In support of that hypothesis, we found that TET1 silencing increased malignant phenotypes, such as cell proliferation and migration (Fig. 3A and B and Supplementary Fig. S2A). Moreover, loss of TET1 increased mesenchymal phenotypes, as shown by upregulation of *ZEB1* and *vimentin (VIM)*, and downregulation of E-cadherin (*CDH1*), in Z172 and Z183 cells (Fig. 3C). Furthermore, *ZEB1* knockdown reversed migration phenotypes induced by TET1 knockdown (Fig. 3D and Supplementary Fig. S2B).

To assess the role of TET1 in malignant transformation, non-tumorigenic immortalized *TET1* knocked-down cells were injected into immunocompromised mice, showing that 1/6 Z172, and 6/6 Z183, *TET1* knockdown cells, and 0/6 and 1/6 control cells, grew tumors, respectively (Fig. 3E–G). To our knowledge, these data provide the first evidence that loss of *TET1* promotes EMT, and transformation, in HPV-immortalized cervical HSIL cells. Such results would also agree with numerous studies confirming that the reverse phenotypic change, the mesenchymal-to-epithelial transition (MET), is required for induced pluripotency [25].

3.4. TET1 suppresses ZEB1 and VIM (vimentin) expression, via 5hmC-independent, histone modification

We found our results of TET1-mediated suppression of EMT quite interesting, as TET1 is widely known as a DNA demethylase, associated with gene activation, and its role in gene suppression was previously unknown. To better understand this phenomenon, we assessed possible direct binding of TET1 to gene promoters, by ChIP-qPCR. These results confirmed that indeed, TET1 and 5hmC cooccupied the promoters of *ZEB1* and *VIM* (Fig. 4A–C, 4F–H), both of which were downregulated in *TET1* knocked-down cells, while analogously, loss of TET1 correlated with loss of 5hmC, within the two gene promoters (Fig. 4C and H).

These 5hmC promoter occupancy results directly contradict the widely held view of this chromatin modification as a marker of gene activation. Consequently, we examined other markers of the “histone code” [26]. Those assays showed that *TET1* knockdown associated with the activating histone mark, trimethylated histone H3 lysine 4 (H3K4me3), while concomitantly decreasing the repressive histone mark, H3K27me3 (Fig. 4D and E, 4I–J), in both Z172 and Z183 cells. These results suggest that TET1 binds to the promoters of *ZEB1* and *VIM*, inhibiting their expression through well-established histone code marks, but independent of DNA demethylation. To elucidate possible TET1-interacting gene-repressive histone marks, loss of H3K4Me3, and gain of H3K27Me3, we assessed *ZEB1* promoter binding by the H3K4 demethylase, LSD1, and the H3K27 trimethyltransferase, EZH2, (Fig. 5A and B). Moreover, coimmunoprecipitation assays confirmed that TET1, LSD1, and EZH2 interact with each other to form a transcriptionally repressive complex (Fig. 5C), while IHC staining revealed upregulation of LSD1 and EZH2, during tumor progression from normal epithelia to HSIL, similar to TET1 (Fig. 5D and E). Moreover, EZH2 showed an expression pattern similar to TET1, i.e., being downregulated in invasive cancer (The median of EZH2 level in normal squamous epithelium, LSIL, HSIL and SCC are 0, 20, 120, 10, respectively.), although LSD1 remained highly expressed in the latter (The median of LSD1 level in normal squamous epithelium, LSIL, HSIL and SCC are 50, 100, 160, 120, respectively.). Intriguingly, these results indicate a 5hmC-independent function of TET1, which interacts with LSD1 and EZH2 to form a trimeric chromatin-modifying, silencing complex that acts on the *ZEB1* and *VIM* promoters, to suppress their expression and inhibit EMT. Thus, TET1 silencing promotes EMT, and

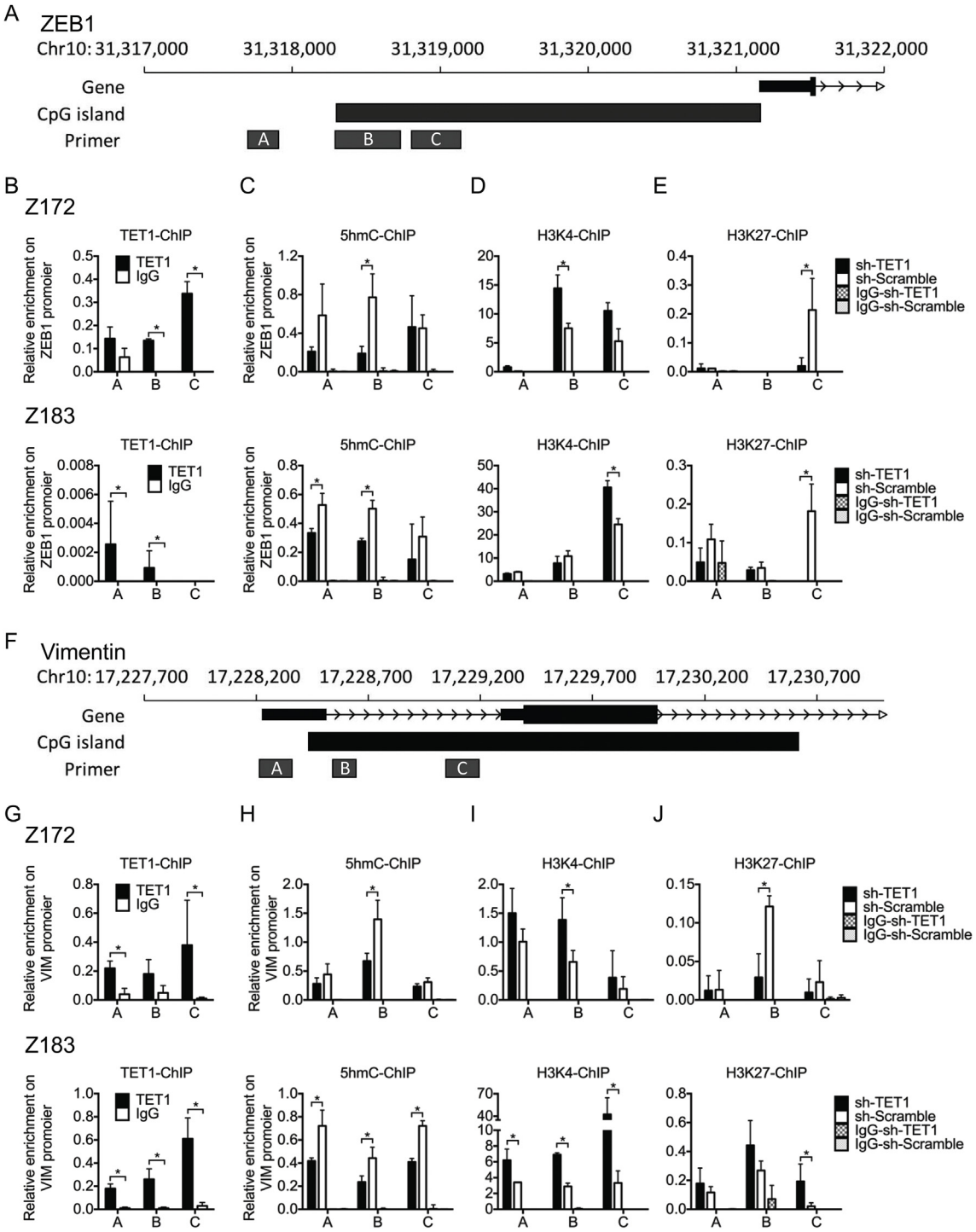

Fig. 4. TET1 silences the mesenchymal marker genes *ZEB1* and *VIM*, through histone modification. The location of ChIP-PCR primers in the promoters of *ZEB1* (A) and *VIM* (E). Anti-TET1 ChIP and anti-5hmC of *ZEB1* (B–D) and *VIM* (F–H). TET1 binding to the *ZEB1* (B) and *VIM* (F) promoters. *TET1* knockdown reduced levels of 5hmC in the *ZEB1* (C) and *VIM* (H) promoters. *TET1* siRNA knockdown decreased levels of H3K4me3, an activating histone mark, while increasing levels of H3K27me3, a repressive histone mark, in the *ZEB1* (D–E) and *VIM* (I–J) promoters. On the other hand, levels of H3K27me3, in the *ZEB1* (E) and *VIM* (J) promoters, decreased in scramble-siRNA-transfected control cells.

transformation, in HPV-immortalized cells.

4. Discussion

Ten-eleven translocation methylcytosine dioxygenase 1 (TET1) is well known to associate with stemness phenotypes in embryonic stem and induced pluripotent cells [17,27]. However, its role across the many steps of tumorigenesis remains little known. In the present study, we showed that TET1 promotes stemness, and inhibits EMT, in HPV-immortalized precancerous cells, via both 5hmC-dependent and -independent mechanisms (Fig. 6A). In particular, TET1 interacted with the well-known chromatin-modifying suppressors, LSD1 and EZH2, in precancerous cervical lesions (High-grade squamous intraepithelial lesion, HSIL). This interaction subsequently silenced the epithelial-to-mesenchymal transition (EMT) markers *ZEB1* and *VIM*, which were not previously associated with TET1 activity (i.e., active demethylation of

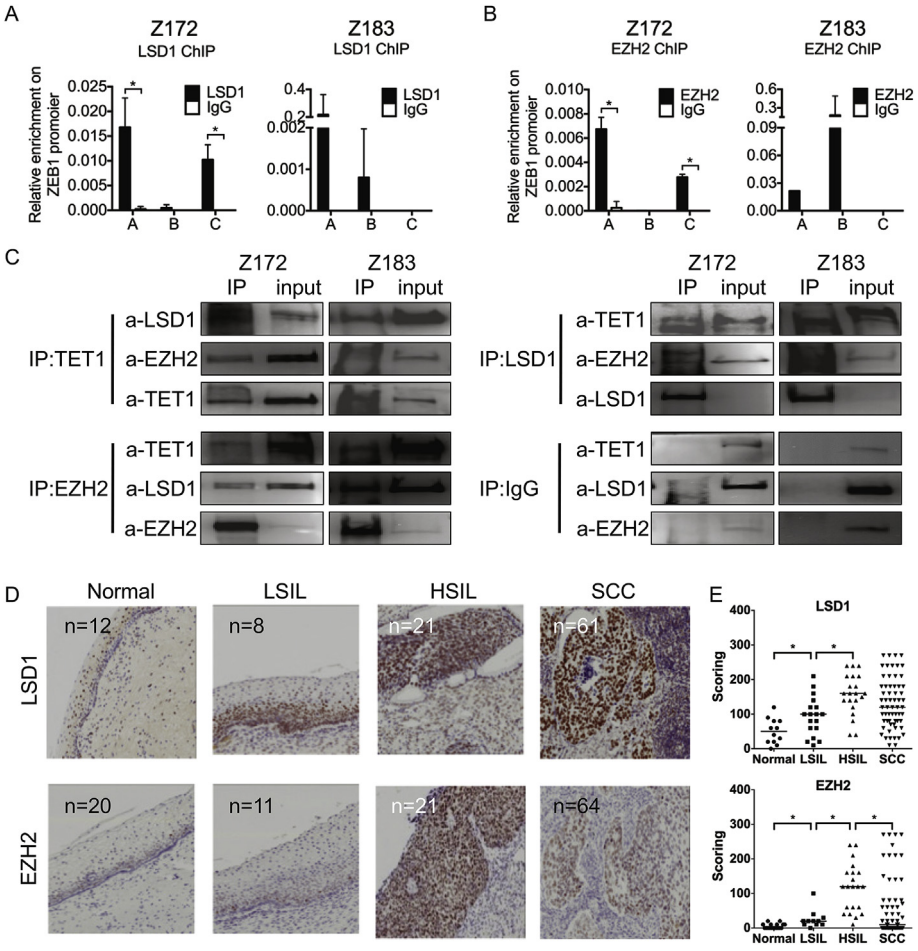

Fig. 5. TET1, LSD1, and EZH2 form a trimeric complex. (A–B) ChIP–qPCR showed that the H3K4Me3 demethylase LSD1 and the H3K27 trimethylase EZH2, both involved in gene repression, were enriched in the *ZEB1* promoter, in precancerous cells. (C) Coimmunoprecipitation showed that TET1, LSD1, and EZH2 directly interact with one another. (D–E) Levels of TET1 and 5hmC in samples across the full spectrum of lesions involved in cervical carcinogenesis.

5-methylcytosine, generating 5hmC) (Fig. 6B).

Although HPV is the major causative factor of cervical cancer [28], its absence or transient expression, in the epithelia as a whole, suggests the necessity of its persistent infection in specific cervical subpopulations (i.e., reserve or stem cells), for the occurrence of full transformation [29]. Reserve cells, residing in the transitional zone between the columnar and squamous epithelium, have been postulated to be stem cells susceptible to HPV infection [30]. In that regard, the HPV "early genes", E6 and E7, can upregulate stemness-related genes, such as *OCT4*, *KLF4*, and *NANOG*, (but not *TET1*) [31,32], thus facilitating reserve cell self-renewal [33]. However, E2, rarely studied and absent in most E6/7 transformation models, can also increase stemness properties [34], as well as TET1 expression [35]. Our use of full-length HPV (including E2)-immortalized cells, in the present study, revealed direct evidence of TET1-mediated upregulation of stemness genes and stemness phenotypes, revealing a role for E2 in TET1-mediated epigenetic reprogramming in HPV-transformed cells. Further assessment of possible association of E2, with TET1 expression/activity, is warranted in future studies.

Although it has been well studied in embryonic stem and induced pluripotent cells, the precise role of TET1 in cancer biology remains controversial. For example, TET1 was earlier reported to be a tumor suppressor, through 5hmC-dependent activation (and thus, demethylation) of tumor suppressor genes, such as the tissue inhibitors of metalloproteinase 2/3 (*TIMP2/3*), in prostate and breast cancers [36], homeobox A9 (*HOXA9*), in breast cancer [37], and *DKK* and *SFRP2*, in ovarian and colon cancer [38,39]. On the other hand, TET1 was also reported to demethylate and activate cancer stemness mediators in neuroblastoma, glioblastoma, and breast cancer, during hypoxia [40–42], suggesting a tumor-promoting role. However, to our knowledge, a 5hmC-independent role for TET1 has never been reported. Our results herein demonstrate both 5hmC-dependent and -independent roles for TET1, in HPV-immortalized cervical HSIL cells, with TET1 5hmC-dependent demethylation of stemness genes being consistent with previous reports [41].

EMT is important for cancer stemness, and the two processes have even been hypothesized to be one and the same. In particular, expression of mesenchymal transcription factors promotes EMT, and de-differentiates epithelial cancer cells to a stem-cell-like phenotype [43]. However, growing evidence shows that EMT may not necessarily associate with stemness [44], especially in nonmalignant tissue stem cells [45]. For example, embryonic stem cells (ESCs) are epithelial-like, and the reverse process, the mesenchymal-to-epithelial transition (MET) is required for the reprogramming of induced pluripotent stem cells [46]. In cancer, downregulation of EMT inducers is necessary during metastatic colonization, with stemness required for secondary site tumorigenesis [44]. Moreover, it has now been speculated that the EMT/MET/stemness phenotypes exist on a continuum, with various hybrid states controlled by a miR-200/ZEB1/Lin28 feedback loop [47]. It has also been reported that loss of paired related homeobox protein 1 (PRXX1, which interacts with Twist1) uncouples EMT from stemness [44]. Thus, interesting future studies could study possible "crosstalk" between TET1, PRXX1, ZEB1, and the miR-200 regulators of EMT.

Our previous report demonstrated that cancer stem cells with

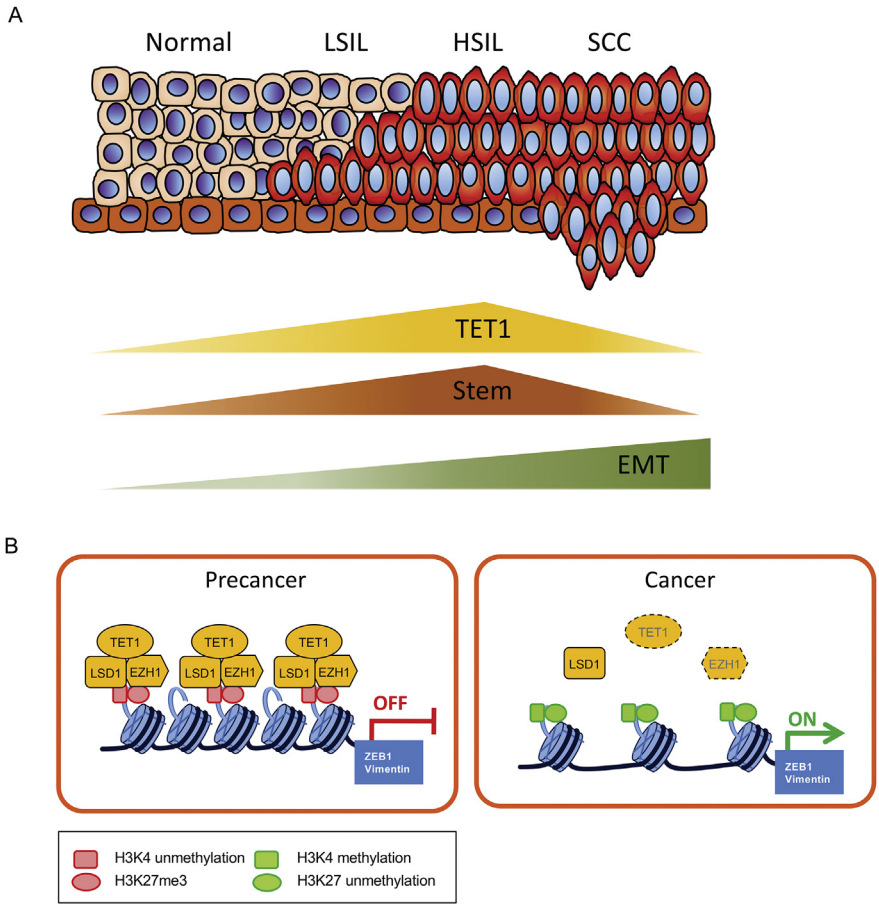

Fig. 6. A proposed model for TET1 in cervical cancer progression. (A) TET1 promotes stemness properties, and inhibits EMT, in precancerous stages. (B) A novel postulate is that a 5hmC-independent function of TET1, i.e., downregulation of *ZEB1* and *VIM*, through its interaction with the chromatin modifiers, LSD1 and EZH2, inhibits EMT.

multiple-lineage differentiation ability upregulate epithelial markers, and downregulate mesenchymal markers, compared to cancer progenitor cells with limited-lineage differentiation ability [48]. In this study, we showed that TET1 maintains stemness, and inhibits EMT, in HSIL. Although the mechanism of TET1 downregulation, during full cancer transformation, remains unclear, investigation of its regulation in invasive cancer may provide insight into the transition from HPV infection to LSIL to HSIL to invasion in cervical cancer.

The significance of TET1 to EMT is interesting. Previously, it was shown that TET1 correlates with epithelial phenotypes in hepatocellular [49] and lung cancer [50]. However, the possibility of TET1 as an EMT inhibitor has remained largely unexplored. In this study, we found that TET1 interacts with well established chromatin modifiers, such as the trimethylated histone H3, lysine 4 (H3K4me3) demethylase, LSD, and the H3K27 trimethyltransferase EZH2, to suppress mesenchymal genes such as *ZEB1* and *VIM*, upregulate the epithelial gene *CDH1*, and prevent tumor progression of dysplastic HSIL. Analogously, *TET1* silencing associated with transformation of HPV-immortalized cells to become tumorigenic, as xenografting of $TET1^{KD}$ HSIL cells resulted in tumor formation, suggesting a unique "guardian" role, for TET1, against HPV-related carcinogenesis. This 5hmC-independent suppression of EMT, via well-known histone modifications, sheds a new light on TET1 in cancer biology.

In summary, we herein demonstrate both 5hmC-dependent and -independent roles of TET1 in HPV-mediated carcinogenesis, providing greater insight into our understanding of epigenomic alterations in the etiology of this gynecological cancer. Further investigations of epigenetics, in the natural history of LSIL lesions, may reveal useful biomarkers for predicting tumor progression in cervical and other cancers.

Author contributions

HC Lai conceived the project, interpreted the results, and wrote the manuscript. PH Su and YW Hsu designed and performed the experiments, analyzed the data, and prepared the manuscript. RL Huang conducted the bioinformatics analyses. TK Chao contributed to clinical sample collection. LY-Chen, CC Liao, CW Chen, TI Wu, SP Mao and C Balch edited the manuscript.

Conflicts of interest

The authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.01.033.

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