Cellular Physiology and Biochemistry

Cell Physiol Biochem 2019;53:948-960
DOI: 10.33594/000000188
Published online: 11 December 2019
Accepted: 5 December 2019

Original Paper

HOTAIR Knockdown Decreased the Activity Wnt/β-Catenin Signaling Pathway and Increased the mRNA Levels of Its Negative Regulators in Hela Cells

Eric Genaro Salmerón-Bárcenas[a,b] Berenice Illades-Aguiar[b]
Oscar del Moral-Hernández[c] Arturo Ortega-Soto[d]
Daniel Hernández-Sotelo[a]

[a]Laboratorio de Epigenética del Cáncer, Facultad de Ciencias Químico Biológicas/Universidad Autónoma de Guerrero, Chilpancingo de los Bravo, México, [b]Laboratorio de Biomedicina Molecular, Facultad de Ciencias Químico Biológicas/Universidad Autónoma de Guerrero, Chilpancingo de los Bravo, México, [c]Laboratorio de Virología del Cáncer, Facultad de Ciencias Químico Biológicas/Universidad Autónoma de Guerrero, Chilpancingo de los Bravo, México, [d]Departamento de Toxicología, Centro de Investigación y de Estudios Avanzados/Instituto Politécnico Nacional, Ciudad de México, México

Key Words
Wnt/β-catenin • HOTAIR • Methylation • TET1 and hydroxymethylation

Abstract
Background/Aims: HOTAIR is a long non-coding RNA that promotes the development of human cancer. TET1 enzyme is involved in DNA demethylation by oxidation of 5-methylcytocine and it is considered a tumor suppressor in some types of cancer. HOTAIR and TET1 are involved in modulation of the Wnt/β-catenin signaling pathway, but their role in cervical cancer remains to be elucidated. The aim of this work was to analyze the effect of HOTAIR in TET1 expression, Wnt/β-catenin signaling, and expression, methylation and hidroxymethylation of some negative regulators of this pathway in HeLa cells. ***Methods:*** HOTAIR and TET expression were analyzed by RT-qPCR and western blot. The HOTAIR knockdown was done with DsiRNA and the activity of the Wnt/β-catenin signaling pathway through luciferase assays and β-catenin nuclear translocation. The mRNA levels of SNAIL, EDN3, CYCD1, SPRY2 (targets of Wnt/β-catenin pathway) PCDH10, SOX17, AJAP1, and MAGI2 (negative regulators of Wnt/β-catenin pathway) were evaluated by RT-qPCR. The DNA methylation and hidroxymethylation of negative regulators of the Wnt/β-catenin pathway were evaluated by methylation-specific PCR and chemical modification, followed by digestion and quantitative PCR. ***Results:*** HOTAIR knockdown in HeLa cells decreased the activity of Wnt/β-catenin signaling pathway. It

Daniel Hernández-Sotelo
Laboratorio de Epigenética del Cáncer, Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Guerrero, Avenida Lázaro Cárdenas S/N Cuidad Universitaria, Chilpancingo de los Bravo, Guerrero, 39070 (México)
Tel. 52 7474710901, Fax 52 7474710901, E-Mail dhernandez@uagro.mx

increased the mRNA levels of Wnt/ β-catenin negative regulators through a decrease in their promoter's methylation pattern. TET1 enzyme was also down-regulated in HOTAIR knockdown cells. ***Conclusion:*** Our study suggests a mechanism in which HOTAIR promotes the over-activation of Wnt/β-catenin signaling pathway by downregulation of PCDH10, SOX17, AJAP1 and MAGI2 and also TET.

Introduction

HOX transcript antisense intergenic RNA (HOTAIR) is a well-characterized long non-coding RNA that interacts with the Polycomb repressive complex 2 (PRC2) [1] and lysine-specific demethylase 1 (LSD1) [2] to modulate the expression of genes associated with the progression of cancer [3]. HOTAIR over-expression has been reported in several types of human cancer, which induce the proliferation, migration, invasion, metastasis and apoptosis inhibition [4-5]. Furthermore, HOTAIR triggers the Wnt/β-catenin pathway in Lewis lung cancer cell [6], esophageal squamous cell carcinoma [7], pancreatic ductal adenocarcinoma [8], gastric [9] and ovarian cancer [10]. However, the molecular mechanisms involved in this stimulation has not been fully elucidated.

Ten-eleven translocation 1 (TET1) is a dioxygenase that depends of Fe (II) and 2-oxoglutarate cofactors. This enzyme is involved in DNA demethylation by oxidation of 5-methylcytocine (5mC) to 5-hydroxymetylcytosine (5hmC), 5-formylcytosine (5fmC) and 5-carboxylcytosine (5cmC) [11]. TET1 is mainly regulated at transcriptional level by HIF-2α [12], Oct4, SOX2 [13], PRC2 [14], DNA methylation and polyADP-ribosylation [15]. TET1 is involved in many diseases, including several types cancer [16], in which is down-regulated [17-21]. Moreover, TET1 decreases the stimulation of theWnt/β-catenin pathway by de-repression of inhibitors through changes in methylation and hydroxymethylation levels of their promoters in colon [22] and ovarian cancer [23].

The Wnt/β-catenin signaling pathway has a critical role in development, differentiation and tissue homeostasis [24]. Aberrantly, this signaling pathway is widely involved in several human malignancies, including many types of human cancer [25]. The negative regulators of this pathway are frequently altered in human cancer mainly by genetic and epigenetic mechanisms [25-27]. The abnormal methylation is one of the main epigenetic mechanisms for the loss of function of negative regulators of the Wnt/β-catenin pathway [28].

In summary, there are studies that clearly indicate that HOTAIR and TET1 are involved in modulation of the Wnt/β-catenin signaling pathway in human cancer. Nevertheless, the role of HOTAIR and TET1 in the Wnt/β-catenin signaling pathway is unknown in cervical cancer and its cellular lines. Therefore, in this work, we analyzed the HOTAIR effect in TET1 expression, Wnt/β-catenin pathway and expression, methylation and hidroxymethylation of some negative regulators of this pathway in HeLa cells.

Materials and Methods

Cell culture and transfections

HeLa cells were purchased from American Type Culture Collection (ATCC, USA) and cultured in DMEM/F12 1:1 medium (Gibco; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B (Caisson Labs; Smithfield, UT, USA), at 37°C and 5% CO_2. The cells (~500, 000 cells, 12-wells plates) were transfected with 30 µM of DsiRNA targeting HOTAIR (DsiHOTAIR) (IDT; San Diego, CA, USA) or as control DsiCTRL (IDT; San Diego, CA, USA) using Lipofectamine 2000 (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's protocol, then collected 48 hours later for further analysis.

Total RNA and genomic DNA isolation

Total RNA was extracted with TRIzol reagent (Invitrogen; Carlsbad, CA, USA) according to the manufacturer´s protocol. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega; Madison, WI, USA) according to the manufacturer´s instructions. The concentrations of RNA and DNA were determined by spectrophotometry with Nano-Drop 2000c Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA).

RT-qPCR

The mRNA levels of TETs, negative regulators and target genes of Wnt/β-catenin pathway were analyzed by RT-qPCR using the KAPA SYBR FAST One-Step (KapaBiosystem; Boston, MA, USA) according to the manufacturer's protocol. The conditions of reverse transcription and amplification were: 37°C for 30 s, 42°C for 5 min and 95°C for 5 min; 40 cycles of amplification: 5 s at 95°C, 30 s at 60°C and 30 s at 72°C; melt curve: 15 s at 95°C, 1 min at 60°C and 15 s at 95°C. Reactions were performed in QuantStudio 3 (Applied Biosystems; Foster City, CA, USA). Data were normalized using GAPDH as an internal control and relative expression differences were calculated using the $2^{-\Delta\Delta Ct}$ method. Primers sequences are shown in Table 1.

Table 1. Primer sequencies

Gene	Forward	Reverse	Tm °C
RT-qPCR			
HOTAIR	GGTAGAAAAAGCAACCACGAAGC	ACATAAACCTCTGTCTGTGAGTGCC	60
GAPDH	CCGGGAAACTGTGGCGTGATGG	AGGTGGAGGAGTGGGTGTCGCTGTT	60
TET1	GCTGCTGTCAGGGAAATCAT	ACCATCACAGCAGTTGGACA	60
TET2	*CCAATAGGACATGATCCAGG*	*TCTGGATGAGCTCTCTCAGG*	61
TET3	*TCGGAGACACCCTCTACCAG*	*CTTGCAGCCGTTGAAGTACA*	60
PCDH10	CCCGTCTACACTGTGTCCCT	GGAGTACACGACCTCACCGT	60
SOX17	AGTGACGACCAGAGCCAGAC	CCTTAGCCCACACCATGAAA	63
AJAP1	GTTAGCACAACGGAGCCTTC	GATGATCTGATGGACAGCCA	56
MAGI2	TCGGAGGATGGAGTCTGGATTTGGC	GCCCATGGCAATGACAGCTCCAA	60
β-Catenin	TGCAGTTCGCCTTCACTATG	ACTAGTCGTGGAATGGCACC	60
CYCD1	CCGTCCATGCGGAAGATC	ATGGCCAGCGGGAAGA	60
SNAIL	TGACCTGTCTGCAAATGCTC	CAGACCCTGGTTGCTTCAA	60
EDN3	CTATTGCCACCTGGACATCA	GCCGTCCTTGAATTACTGCT	56
SPRY2	TTGCTCGGAAGTTGGTCTAA	TCAGGTCTTGGAAGTGTGGT	42
DNMT1	GGTTCTTCCTCCTGGAGAATG	GTCTGGGCCACGCCGTACTG	60
DNMT3A	GGTGCTGTCTCTCTTTGATG	ATGCTTCTGTGTGACGCTG	60
DNMT3B	ACCACCTGCTGAATTACTCACG	GATGGCATCAATCATCACTGG	60
MSP			
TET1	GTCGGTAGGCGTTTTTCGC	CCCAACTCACCGCTAACCG	62
PCDH10	TCGTTAAATAGATACGTTACGC	TAAAAACTAAAAACTTTCCGCG	60
SOX17	GGAGATTCGCGTAGTTTTCG	AACCCGACCATCACCGCG	60
AJAP1	TTTGGTAGAGTTTTTCGATTCGGTAGC	ACCGAAACTCCGCGCCGATAA	60
MAGI2	CGTAGAGTTCGAGATGTGGTATTAGGC	AAACTCCTATACGAAAAAAACGCGCTA	60
qPCR			
PCDH10	GCGGGGCGGGACAGAACTCT	TTTCCGCGGCATTGTCCCC	64
SOX17	CGGAGGGTTGAGGGGAGCG	GCGCTCTGGGTCTGGCTCTG	63
AJAP1	GCGGCCGTCTGCAGAGCGAG	CTCTGCCAGAGGCTCCGCCG	64
MAGI2	GCCTTCTCCCGCAATGCCC	GCTCTGCGAGCGTCCTGGCG	58

Protein extraction and western blotting

Total protein extraction was performed with RIPA buffer (50 mM Tris-HCl, 1 mMEDTA, 150 mM NaCl, 1mM phenylmethylsulfonylfluoide, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1% NP-40, 0.25% sodium deoxycholate, 10 mM NaF, 1 mM Na_2MoO_4 and 1 mM Na_3VO_4, pH 7.4). Cytoplasmic protein extraction was performed with buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 50 µg/µl complete) followed by nuclear protein extraction using buffer C (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 40 µg/µl complete). Protein concentration was determined with Bradford method and 100 µg (total proteins) or 60 µg (cytoplasmic or nuclear proteins) were separated by SDS-PAGE and transferred to a nitrocellulose membranes. Membranes were blocked with 5% skim milk for 2 hours and incubated with primary antibodies at 4°C overnight. Primary antibodies were

TET1 (GTX627420, 1:500) (GeneTex; Irvine, CA, USA) and β-catenin (GTX101435, 1:1000) (GeneTex; Irvine, CA, USA). Tubulin (GTX114, 1:1000) (GeneTex; Irvine, CA, USA) and Lamin A (SC20681, 1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as a loading controls. Membranes were washed and incubated with secondary antibodies at room temperature for 2 hours. Finally, membranes were washed and immunoreactivity was detected by chemiluminescence using MicroChemi (DNR Bio-imaging System) charge-coupled device (CCD) imager (DNR; MahaleHaHamisha, Jerusalem, Israel). Densitometryc analysis was performed in the ImageJ software (NIH; Bethesda, Maryland, USA).

TOP/FOPflash luciferase assay report

HeLa cells (~500, 000 cells, 12-wells plates) were transiently co-transfected with 500 ng of canonical Wnt reporter vector TOPFlash-Luc or mutant reporter vector FOPFlash-Luc (Upstate Biotechnology Inc; Lake placid, NY, USA) and DsiHOTAIR or DsiCTRL, 48 hours after, cells were harvested and luciferase activity was measured with Luciferase Assay System kit (Promega; Madison WI, USA) in the GloMax Multi Jr Luminometer (Promega; Madison, WI, USA).

Methylation-specific PCR (MSP)

The methylation analysis of TET1, PCDH10, SOX17, AJAP1 and MAGI2 genes was done by MSP. Primers sequences are shown in Table 1. Briefly, 1.5 μg of genomic DNA was modified with sodium bisulfite using the EZ DNA Methylation-Gold™ Kit (Zymo Research; Irvine, CA, USA). MSP was performed using Amplitaq Gold Master Mix (Applied Biosystems; Foster City, CA, USA) according to the manufacturer's protocol. Amplification conditions were: denaturation, 95°C for 10 min; 30 cycles of amplification: 30 s at 95°C, 30 s at 60°C and 30 s at 72°C; a final extension of 72°C for 10 min. Densitometryc analysis was performed with the ImageJ software (NIH; Bethesda, Maryland, USA).

5hmC analysis

The 5hmC analysis of PCDH10, SOX17, AJAP1 and MAGI2 genes was done with the Quest 5hmC Detection Kit (Zymo Research; Irvine, CA, USA). Briefly, a total of 300 ng of genomic DNA was treated with a glucosyltransferase (GT) or unglucosylate (No GT) for 6 hours. The DNA was digested with *MspI* (30 U) at 37°C for 2 hours, followed by column purification with DNA Clean & Concentrator-5 (Zymo Research; Irvine, CA, USA). Finally, 5hmC was quantified by qPCR using primers (Table 1) that flank one *MspI* site (CCGG). The qPCR was performed with qPCR GreenMaster with UNG (Jena Bioscience; Löbstedter, Jena, Germany) according to the manufacturer's protocol. Amplification conditions were: 50°C for 2 min and initial denaturation at 95°C for 2min; 40 cycles of amplification: 15 s at 95°C, 20 s at 60°C and 30 s at 72°C. Reactions were performed in QuantStudio 3 (Applied Biosystems; Foster City, CA, USA). The 5hmC level was calculated according previously reported [29].

Statistical Analysis

All data were analyzed in SigmaPlot 10.0 software (Jandel Scientific; San Rafael, CA, USA). Data were presented as the mean ± standard error. The p value was determined using Student's t-test and $p<0.05$ was considered as statistically significant.

Results

Inverse expression of TET1 and HOTAIR in cervical cancer cell lines

HOTAIR promotes carcinogenesis through the deregulation of tumor suppressor genes and oncogenes. However, its molecular mechanisms have not been fully elucidated. Several studies have shown that HOTAIR is involved in abnormal methylation of tumor suppressor, targeting enzymes involved in DNA methylation (DNMTs). However, it has not been established whether HOTAIR also regulates enzymes involved in demethylation (TETs). To explore the plausible relationship between HOTAIR and TET1, 2 or 3, we determined their expression by RT-qPCR in cervical cell lines. We found that HOTAIR expression is increased in HeLa cells while it is diminished in C-33A and SiHa cells (Fig. 1A). In contrast, TET1 expression is decreased in HeLa cells and increased in C-33A cells (Fig. 1B). Moreover, TET2 expression is increased in C-33A cells (Fig. 1C) and there is no difference on TET3 expression in cervical cell lines (Fig. 1D). In addition, we analyzed HOTAIR and TET1 expression in The Cancer Genome Atlas (TCGA) database and found a negative correlation between HOTAIR and TET1 expression in cervical cancer samples as well as in other cancer cell lines (Supplementary Fig. S1 – for all supplemental material see www.cellphysiolbiochem.com). These results demonstrate that an inverse expression between HOTAIR and TET1 is present in cancer cells, suggesting a plausible role of HOTAIR in TET1 expression.

HOTAIR knockdown increases TET1 expression

To gain insight into the involvement of HOTAIR in TET1 expression, we knocked down HOTAIR in HeLa cells (Fig. 2A) and evaluated the TET1, 2 and 3 expression. Indeed, reduction of HOTAIR expression, led to an increase in TET1 expression (Fig. 2B and C). This effect is specific since neither TET2 nor TET3 mRNA levels were significantly changed in HeLa cells (Supplementary Fig. S2A). Remarkably, it has been reported that DNA methylation of the TET1 promoter decreases its expression in several types of cancer. Therefore, we next

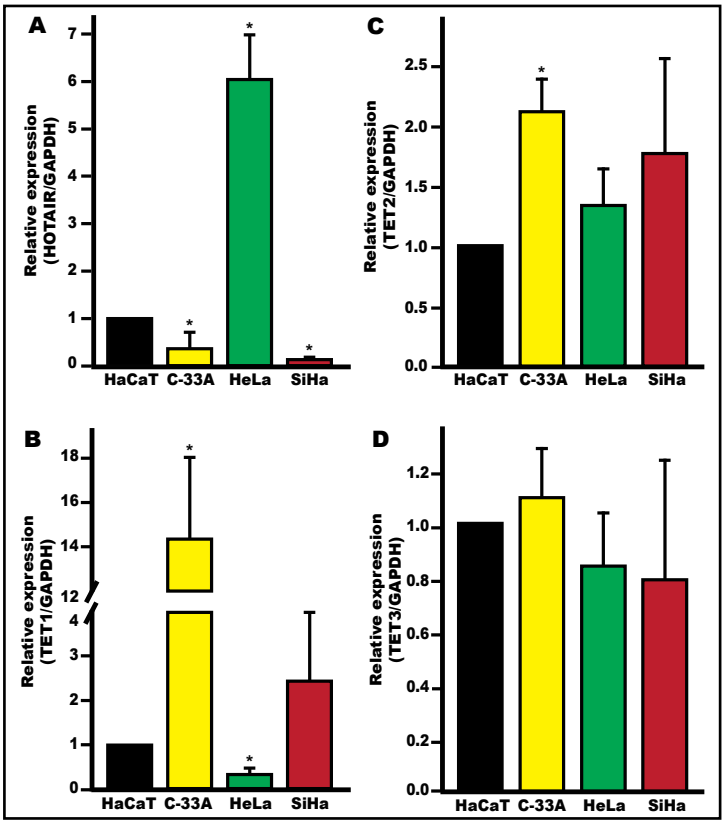

Fig. 1. Inverse expression of HOTAIR and TET1 in cervical cancer cells. HOTAIR (A), TET1 (B), TET2 (C) and TET3 (D) expression was determined in cervical cancer cells by RT-qPCR. The bars represent the mean ± standard deviation from at least three independent experiments. *p<0.05.

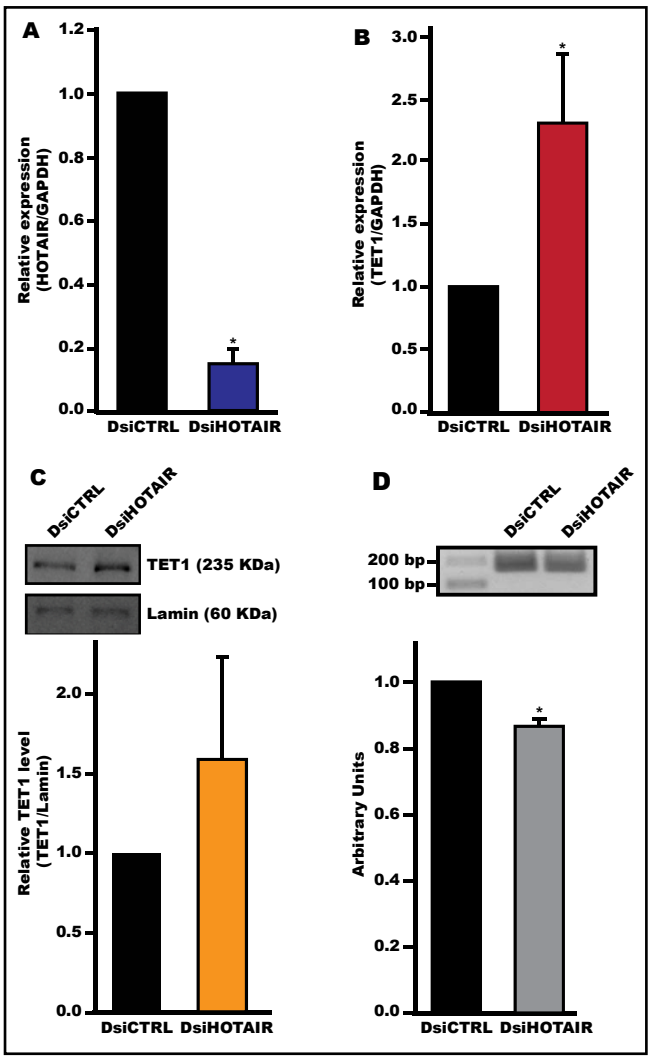

Fig. 2. HOTAIR knockdown increases the TET1 expression in HeLa cells. HeLa cells were transiently transfected with DsiCTRL or DsiHOTAIR and the levels of HOTAIR (A), mRNA (B), protein (C) and promoter methylation (D) of TET1 were determined 48 hours later by RT-qPCR, western blot and MSP, respectively. Three independent experiments were performed. The bars represent the mean ± standard deviation from at least three independent experiments. *p<0.05.

analyzed the effect of HOTAIR knockdown in the methylation status of the TET1 promoter. The results are shown in Fig. 2D, a diminished methylation of the TET1 promoter is present in the absence of HOTAIR. Additionally, we analyzed TET1 expression and methylation in cervical cancer samples of TCGA database and found a negative correlation between TET1 expression and methylation of its promoter (Supplementary Fig. S2B and C). Taken together, these data strongly suggest that HOTAIR downregulates TET1 expression by methylation of its promoter in HeLa cells.

HOTAIR knockdown decreases Wnt/β-catenin pathway in HeLa cells

HOTAIR promotes the stimulation of Wnt/β-catenin pathway while TET1 has the opposite effect on this pathway in cancer cells. Therefore, we determined the transcriptional activity and target genes expression of this pathway, as well as the nuclear translocation of β-catenin upon HOTAIR knockdown. As expected, HOTAIR knockdown significantly decreased the transcriptional activity of the canonical Wnt reporter vector TOPFlash-Luc but not mutant reporter vector FOPFlash-Luc in HeLa cells (Fig. 3A), significantly decreased the expression level of EDN3 and SPRY2, two of the main target genes of this pathway (Fig. 3B) and decreased the nuclear translocation of β-catenin in HeLa cells (Fig. 3C). Also, HOTAIR knockdown does not affect the mRNA level of β-catenin (Supplementary Fig. S3). All these data point out that HOTAIR regulated the Wnt/β-catenin pathway, in part by promoting β-catenin nuclear translocation.

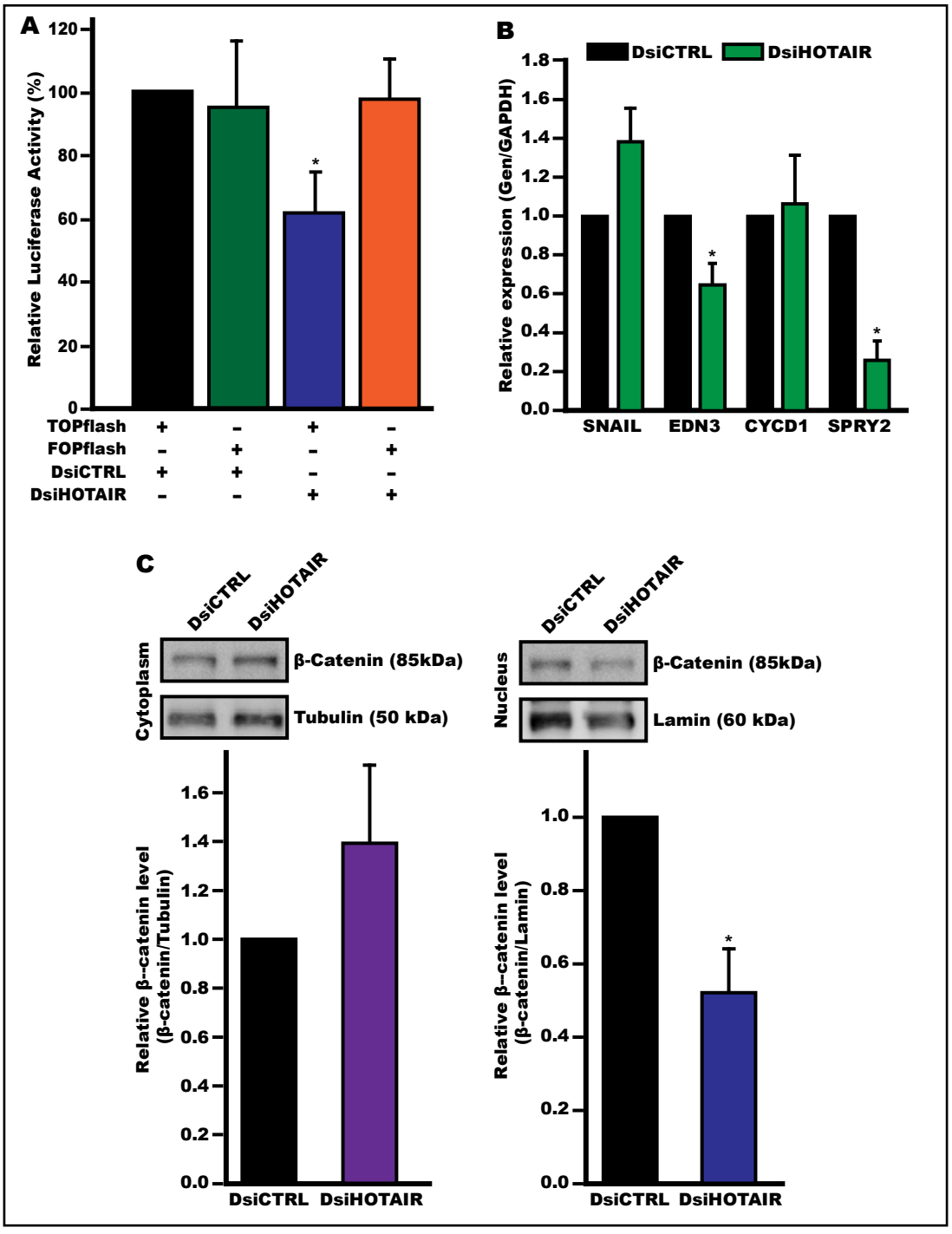

Fig. 3. HOTAIR knockdown decreases the activity Wnt/β-catenin signaling pathway in HeLa cells. HeLa cells were transiently transfected with DsiCTRL or DsiHOTAIR and 500 ng of FOPFlash-Luc (mutant reporter vector) or 500 ng of TOPFlash-Luc (canonical Wnt reporter vector) plasmids and the luciferase activity was determined after 48 (A). mRNA level of SNAIL, EDN3, CYCD1 and SPRY2 was determined by RT-qPCR (B). Representative inmunoblots of β-catenin expression in cytoplasm and nucleus. Tubulin and lamin β were used as loading controls, cytoplasmic and nuclear respectively, below is shown the densitometry analysis (C). The bars represent the mean ± standard deviation from at least three independent experiments. *p<0.05.

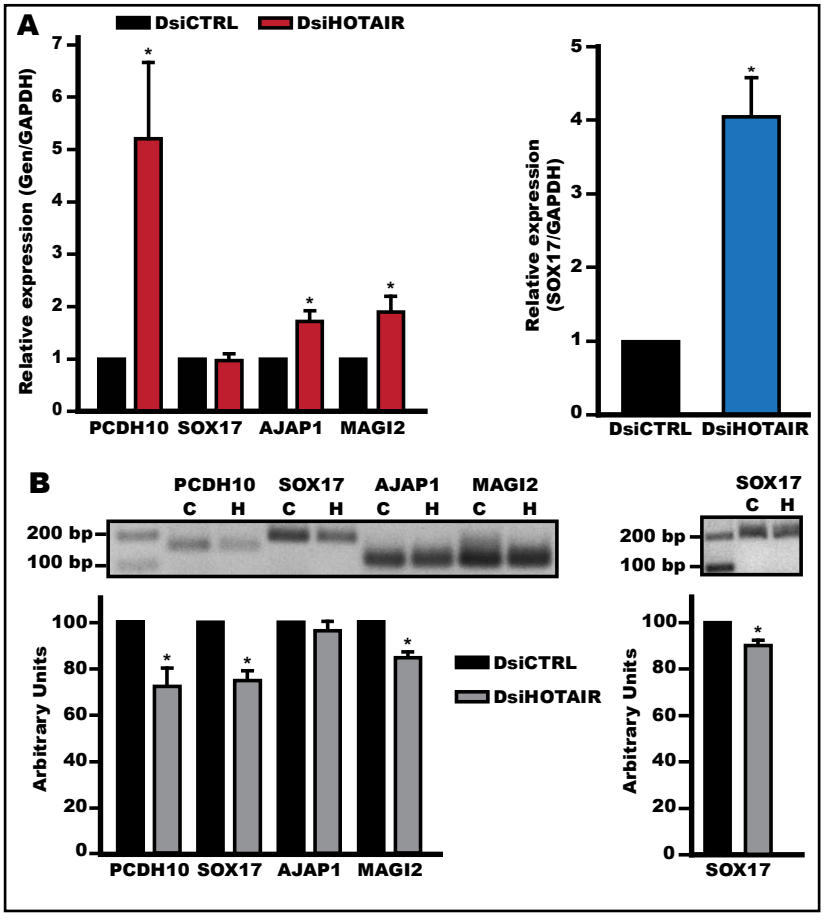

Fig. 4. HOTAIR knockdown increases the mRNA levels of negative regulators of the Wnt/β-catenin signaling pathway maybe by demethylation of theirs promoters in HeLa cells. HeLa cells were transfected with DsiCTRL or DsiHOTAIR, after 48 (left) or 96 (right) hours, the mRNA level of PCDH10, SOX17, AJAP1 and MAGI2 genes was determined by RT-qPCR (A). Analysis of promoter methylation, after 48 (left) or 96 (right) by MSP. Below is shown the densitometry analysis (B). The bars represent the mean ± standard deviation from at least three independent experiments. *p<0.05.

HOTAIR knockdown increases expression of negative regulators of Wnt/β-catenin pathway in HeLa cells

To understand the molecular mechanism by which HOTAIR knockdown decreases Wnt/β-catenin pathway activity, we analyzed the expression of four of its negative regulators: PCDH10, SOX17, AJAP1 and MAGI2. We found that HOTAIR knockdown increased PCDH10, MAGI2, AJAP1 and SOX17 expression at 48 and 96 hours (Fig. 4A). Previously, it has been reported that PCDH10, SOX17, AJAP1 and MAGI2 expression is decreased by methylation of their promoters in cancer. Besides, HOTAIR favors the methylation of tumor suppressor genes promoters, reducing their expression. Therefore, we analyzed the promoter methylation patterns of these genes by MSP. We found that HOTAIR knockdown decreased the methylation of PCDH10, SOX17 and MAGI2 gene promoters (Fig. 4B). Moreover, we analyzed expression and methylation of PCDH10, SOX17, AJAP1 and MAGI2 in cervical cancer samples of the TCGA database. We found a negative correlation between expression and methylation of these genes (Supplementary Fig. S4). Together, these data suggest that HOTAIR decreases PCDH10, SOX17, AJAP1 and MAGI2 expression by methylation of their promoters in HeLa cells.

HOTAIR knockdown increases the levels of 5hmC in SOX17 and MAGI2 promoters

TET1 re-expression leads to the expression of some negative regulators of Wnt/β-catenin pathway through the increase of 5hmC in their promoters. As described above, HOTAIR knockdown increased TET1 expression, thus we hypothesize that TET1 could be involved in the re-expression of PCDH10, SOX17, AJAP1 and MAGI2 genes through via an augmentation of 5hmC content in their promoters. To this end, we analyzed the levels of

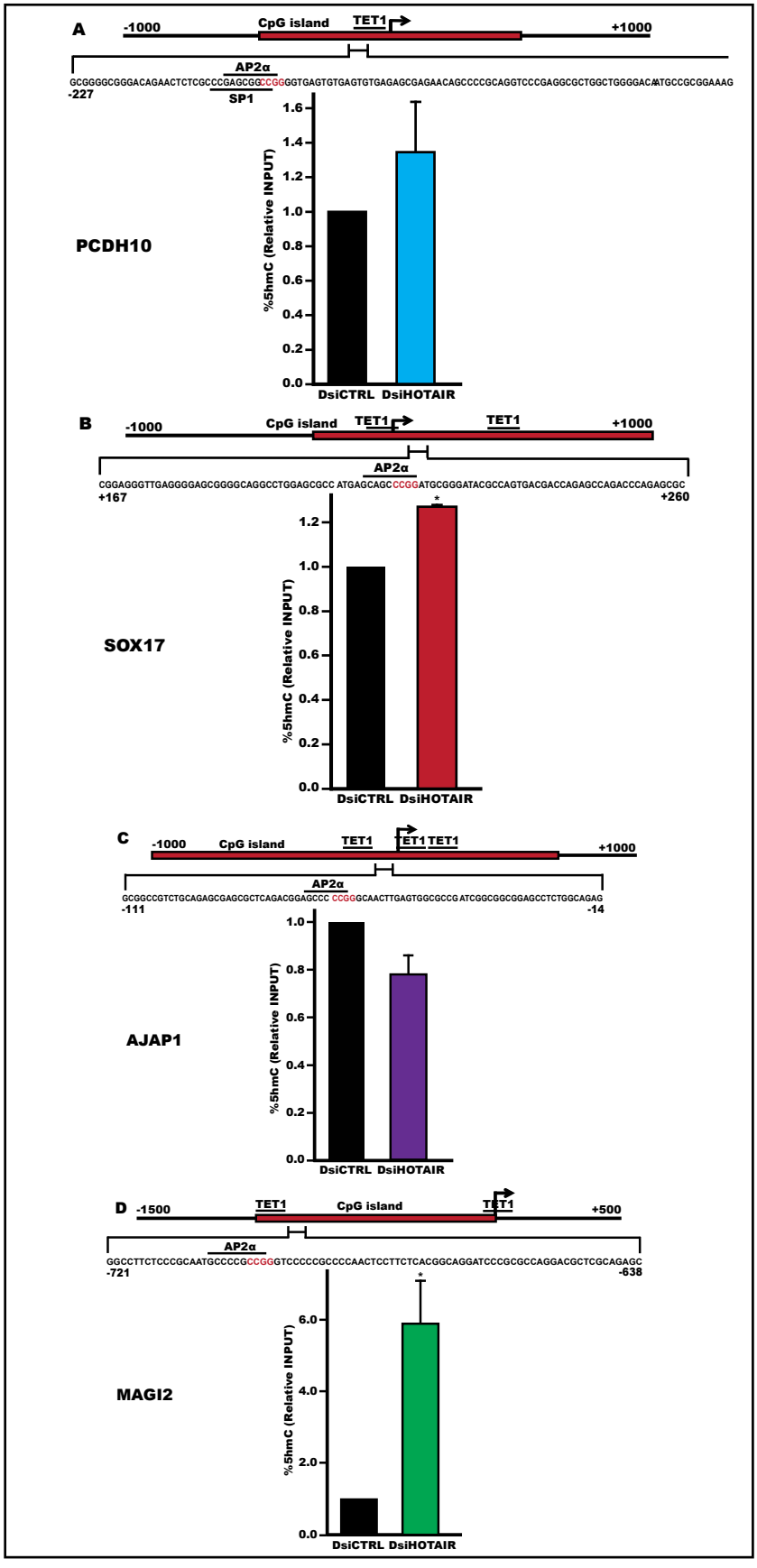

Fig. 5. HOTAIR knockdown increases the 5hmC levels in SOX17 and MAGI2 promoters in HeLa cells. HeLa cells were transiently transfected with DsiCTRL or DsiHOTAIR, and the hydroxymethylation level in PCDH10 (A), SOX17 (B), AJAP1 (C) and MAGI2 (D) promoters was determined 48 hours later as described in the methodology. For each gene shown: the transcription start site (black arrow), CpG island (red box), TET1 binding sites (black line), the amplified region in the qPCR, CCGG site (red sequence) and a binding site for transcription factors related to 5hmC. The bars represent the mean ± standard deviation from at least three independent experiments. *p<0.05.

5hmC in the regulatory regions of PCDH10, SOX17, AJAP1 and MAGI2 genes. As shown in Fig. 5, HOTAIR knockdown increases 5hmC content in SOX17 (5B) and MAGI2 (5D) genes and did not affect significantly the level of 5hmC in PCDH10 (5A) and AJAP1 (5C) genes. These data demonstrate that HOTAIR knockdown increases 5hmC in SOX17 and MAGI2 promoters most possibly, through TET1.

Discussion

HOTAIR lncRNAs has been traditionally related to the development of human cancer. In general, it has been assumed that this non-coding RNA reprograms chromatin organization by relocalization of Polycomb complex and H3K27me3 [3]. TET1 downregulation and reduced levels of specific and global 5hmC are a common feature of human cancer [30]. The Wnt/β-catenin signaling pathway has a critical role in development, differentiation and tissue homeostasis [24]. Aberrantly, this signaling pathway is widely involved in several human malignancies [25]. On the other hand, both HOTAIR and TET1 are implicated in modulation of the Wnt/β-catenin signaling pathway [22-23, 31-32]. Also, HOTAIR specifically in HeLa cells has an important role in cell proliferation, migration, and invasion [33-35].

In this work, we analyzed the participation of HOTAIR in TET1 expression, Wnt/β-catenin pathway and expression, methylation and hidroxymethylation of some negative regulators of this pathway in HeLa cells. We found that HOTAIR knockdown decreased the activity of Wnt/β-catenin signaling pathway. This result has already been reported in lung, esophageal, pancreatic, gastric and ovarian human cancer [6, 8-10, 36]. It is quite possible that the decrease of Wnt/β-catenin signaling activity is the result, at least in part, of the TET1 re-expression, consequence of the demethylation of its promoter in the reduced levels of HOTAIR. Consistent with our results, several studies have found that TET1 is silenced by hypermethylation in cancer [17-21] and that it has a role as negative regulator of the Wnt/β-catenin signaling pathway in nasopharyngeal, colorectal and ovarian human cancer [22-23, 37]. In our results, the HOTAIR knockdown increased the mRNA level of TET1 but the demethylation of its promoter was very small. It is possible that the upregulation of TET1 also can be explained by a reduction of H3K27 methylation (repressive histone marks), a consequence of the EZH2 relocalization via HOTAIR knockdown, as previously has been reported [3, 38]. In this work, the HOTAIR knockdown not decreased the expression of SNAIL and CYCD1. The SNAIL protein is over-expressed in cervical cancer and HeLa cells [39] and CYCD1 protein is coexpressed with p16 in cervical cancer cells with HPV16 infection [40]. Previously has been reported that E6 HPV18 and YB-1 (Y box-binding protein 1) are positive regulators of SNAIL in HeLa cells [39, 41]. In the other hand, has been reported that CYCD1 expression is indirectly modulated by miR-195, miR-2861, HPV16 and 18 in cervical cancer cells [42-43]. The above may be the reason for the absence of a decrease in the expression of SNAIL and CYCD1 in HeLa cells with HOTAIR knockdown.

We also found that HOTAIR knockdown increased the mRNA levels and decreased the promoter methylation in PCDH10, SOX17, AJAP1 and MAGI2 genes (Wnt/β-catenin pathway negative regulators); events that undoubtedly contribute to the decrease of the activityof Wnt/β-catenin signaling. In this regard, previous studies have shown that these genes can be down-regulated by promoter methylation [44-46] in cancer and also other negative regulators of Wnt/β-catenin pathway are inactivated by hypermethylation of its promoter in several types of human cancer [28]. Furthermore, it has been recently reported that TET1 suppresses the activity of Wnt/β-catenin signaling by increasing the levels of 5hmC and decreasing 5mC in promoters of its negative regulators [22-23, 37]. In this context, we detected that HOTAIR knockdown augmented the levels of 5hmC in MAGI2 and SOX17, but not in AJAP1 and PCDH10 genes. Previously, it has been reported that TET1 binds to the SOX17 promoter, decreasing its methylation and increasing its expression in HEK293T and embryonic stem cells [47-48]. We also found that within the MAGI2 promoter, by *in silico* analysis, several TET1 putative binding sites are present adjacent to the region in which the

5hmC content was analyzed [48]. In the case of AJAP1 and PCDH10 promoters, we did not detect a significant increase of 5hmC, but we did find an increase of their mRNA levels and a decrease in methylation. Thus, it is quite possible that the levels of 5hmC are increased in promoter regions not analyzed. Another possible explanation is that TET1 is capable of rapidly converting 5mC into 5hmC and after to 5fmC and 5cmC [49], events that make it difficult to detect high levels of 5hmC. Additional analysis of 5hmC in broader regions of these promoters are compulsory.

Conclusion

In conclusion, our results indicate that the HOTAIR promotes the activity of the Wnt/β-catenin signaling cascade through the increase in the methylation pattern and expression decrease of PCDH10, SOX17, AJAP1 and MAGI2 genes that results from its involvement in TET1 downregulation.

Acknowledgements

We thank Juan Francisco Leyva-Illades for editing the manuscript. This study was supported by grant from CONACYT (242812), México.

Author Contributions

EGSB and OdH designed and performed the experiments. AOS, BIA and DHS conceived and designed the study. BIA and DHS wrote manuscript. All authors read and approved the manuscript.

Disclosure Statement

The authors declare that they have no competing interests.

References

1 Wu L, Murat P, Matak-Vinkovic D, Murrell A, Balasubramanian S: Binding interactions between long noncoding RNA HOTAIR and PRC2 proteins. Biochemistry 2013;52:9519-9527.
2 Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, Shi Y, Segal E, Chang HY: Long noncoding RNA as modular scaffold of histone modification complexes. Science 2010;329:689-693.
3 Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, Wang Y, Brzoska P, Kong B, Li R, West RB, van de Vijver MJ, Sukumar S, Chang HY: Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 2010;464:1071-1076.
4 Deng Q, Sun H, He B, Pan Y, Gao T, Chen J, Ying H, Liu X, Wang F, Xu Y, Wang S: Prognostic value of long non-coding RNA HOTAIR in various cancers. PLoS One 2014;9:e110059.
5 Loewen G, Zhuo Y, Zhuang Y, Jayawickramarajah J, Shan B: lincRNA HOTAIR as a novel promoter of cancer progression. J Can Res Updates 2014;3:134-140.
6 Chen J, Shen Z, Zheng Y, Wang S, Mao W: Radiotherapy induced Lewis lung cancer cell apoptosis via inactivating beta-catenin mediated by upregulated HOTAIR. Int J Clin Exp Pathol 2015;8:7878-7886.
7 Padua Alves C, Fonseca AS, Muys BR, de Barros ELBR, Burger MC, de Souza JE, Valente V, Zago MA, Silva WA, Jr.: Brief report: The lincRNA Hotair is required for epithelial-to-mesenchymal transition and stemness maintenance of cancer cell lines. Stem Cells 2013;31:2827-2832.
8 Jiang Y, Li Z, Zheng S, Chen H, Zhao X, Gao W, Bi Z, You K, Wang Y, Li W, Li L, Liu Y, Chen R: The long non-coding RNA HOTAIR affects the radiosensitivity of pancreatic ductal adenocarcinoma by regulating the expression of Wnt inhibitory factor 1. Tumour Biol 2016;37:3957-3967.

9 Su C, Li J, Cao Y, Xiao L, Shi C: Long noncoding RNA HOTAIR promotes cisplatin resistance in gastric cancer through promoting WIF-1 promoter methylation. Int J Clin Exp Med 2017;10:2717-2726.
10 Li J, Yang S, Su N, Wang Y, Yu J, Qiu H, He X: Erratum to: Overexpression of long non-coding RNA HOTAIR leads to chemoresistance by activating the Wnt/beta-catenin pathway in human ovarian cancer. Tumour Biol 2015;36:9093-9094.
11 Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y: Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 2011;333:1300-1303.
12 Tsai YP, Chen HF, Chen SY, Cheng WC, Wang HW, Shen ZJ, Song C, Teng SC, He C, Wu KJ: TET1 regulates hypoxia-induced epithelial-mesenchymal transition by acting as a co-activator. Genome Biol 2014;15:513.
13 Koh KP, Yabuuchi A, Rao S, Huang Y, Cunniff K, Nardone J, Laiho A, Tahiliani M, Sommer CA, Mostoslavsky G, Lahesmaa R, Orkin SH, Rodig SJ, Daley GQ, Rao A: Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. Cell Stem Cell 2011;8:200-213.
14 Neri F, Incarnato D, Krepelova A, Dettori D, Rapelli S, Maldotti M, Parlato C, Anselmi F, Galvagni F, Oliviero S: TET1 is controlled by pluripotency-associated factors in ESCs and downmodulated by PRC2 in differentiated cells and tissues. Nucleic Acids Res 2015;43:6814-6826.
15 Ciccarone F, Valentini E, Bacalini MG, Zampieri M, Calabrese R, Guastafierro T, Mariano G, Reale A, Franceschi C, Caiafa P: Poly(ADP-ribosyl)ation is involved in the epigenetic control of TET1 gene transcription. Oncotarget 2014;5:10356-10367.
16 Tan L, Shi YG: Tet family proteins and 5-hydroxymethylcytosine in development and disease. Development 2012;139:1895-1902.
17 Cimmino L, Dawlaty MM, Ndiaye-Lobry D, Yap YS, Bakogianni S, Yu Y, Bhattacharyya S, Shaknovich R, Geng H, Lobry C, Mullenders J, King B, Trimarchi T, Aranda-Orgilles B, Liu C, Shen S, Verma AK, Jaenisch R, Aifantis I: Erratum: TET1 is a tumor suppressor of hematopoietic malignancy. Nat Immunol 2015;16:889.
18 Frycz BA, Murawa D, Borejsza-Wysocki M, Marciniak R, Murawa P, Drews M, Kolodziejczak A, Tomela K, Jagodzinski PP: Decreased expression of ten-eleven translocation 1 protein is associated with some clinicopathological features in gastric cancer. Biomed Pharmacother 2014;68:209-212.
19 Lian CG, Xu Y, Ceol C, Wu F, Larson A, Dresser K, Xu W, Tan L, Hu Y, Zhan Q, Lee CW, Hu D, Lian BQ, Kleffel S, Yang Y, Neiswender J, Khorasani AJ, Fang R, Lezcano C, Duncan LM, et al.: Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. Cell 2012;150:1135-1146.
20 Liu C, Liu L, Chen X, Shen J, Shan J, Xu Y, Yang Z, Wu L, Xia F, Bie P, Cui Y, Bian XW, Qian C: Decrease of 5-hydroxymethylcytosine is associated with progression of hepatocellular carcinoma through downregulation of TET1. PLoS One 2013;8:e62828.
21 Yang H, Liu Y, Bai F, Zhang JY, Ma SH, Liu J, Xu ZD, Zhu HG, Ling ZQ, Ye D, Guan KL, Xiong Y: Tumor development is associated with decrease of TET gene expression and 5-methylcytosine hydroxylation. Oncogene 2013;32:663-669.
22 Neri F, Dettori D, Incarnato D, Krepelova A, Rapelli S, Maldotti M, Parlato C, Paliogiannis P, Oliviero S: TET1 is a tumour suppressor that inhibits colon cancer growth by derepressing inhibitors of the WNT pathway. Oncogene 2015;34:4168-4176.
23 Duan H, Yan Z, Chen W, Wu Y, Han J, Guo H, Qiao J: TET1 inhibits EMT of ovarian cancer cells through activating Wnt/beta-catenin signaling inhibitors DKK1 and SFRP2. Gynecol Oncol 2017;147:408-417.
24 MacDonald BT, Tamai K, He X: Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell 2009;17:9-26.
25 Clevers H, Nusse R: Wnt/beta-catenin signaling and disease. Cell 2012;149:1192-1205.
26 Clevers H: Wnt/beta-catenin signaling in development and disease. Cell 2006;127:469-480.
27 Ochoa-Hernandez AB, Juarez-Vazquez CI, Rosales-Reynoso MA, Barros-Nunez P: [WNT-beta-catenin signaling pathway and its relationship with cancer]. Cir Cir 2012;80:389-398.
28 Ying Y, Tao Q: Epigenetic disruption of the WNT/beta-catenin signaling pathway in human cancers. Epigenetics 2009;4:307-312.
29 de la Rica L, Rodriguez-Ubreva J, Garcia M, Islam AB, Urquiza JM, Hernando H, Christensen J, Helin K, Gomez-Vaquero C, Ballestar E: PU.1 target genes undergo Tet2-coupled demethylation and DNMT3b-mediated methylation in monocyte-to-osteoclast differentiation. Genome Biol 2013;14:R99.
30 Huang Y, Rao A: Connections between TET proteins and aberrant DNA modification in cancer. Trends Genet 2014;30:464-474.

31 Guo X, Xiao H, Guo S, Li J, Wang Y, Chen J, Lou G: Long noncoding RNA HOTAIR knockdown inhibits autophagy and epithelial-mesenchymal transition through the Wnt signaling pathway in radioresistant human cervical cancer HeLa cells. J Cell Physiol 2019;234:3478-3489.
32 Xiao Z, Qu Z, Chen Z, Fang Z, Zhou K, Huang Z, Guo X, Zhang Y: LncRNA HOTAIR is a Prognostic Biomarker for the Proliferation and Chemoresistance of Colorectal Cancer via MiR-203a-3p-Mediated Wnt/ss-Catenin Signaling Pathway. Cell Physiol Biochem 2018;46:1275-1285.
33 Kim HJ, Lee DW, Yim GW, Nam EJ, Kim S, Kim SW, Kim YT: Long non-coding RNA HOTAIR is associated with human cervical cancer progression. Int J Oncol 2015;46:521-530.
34 Zhang Y, Cheng X, Liang H, Jin Z: Long non-coding RNA HOTAIR and STAT3 synergistically regulate the cervical cancer cell migration and invasion. Chem Biol Interact 2018;286:106-110.
35 Zheng P, Yin Z, Wu Y, Xu Y, Luo Y, Zhang TC: LncRNA HOTAIR promotes cell migration and invasion by regulating MKL1 via inhibition miR206 expression in HeLa cells. Cell Commun Signal 2018;16:5.
36 Ge XS, Ma HJ, Zheng XH, Ruan HL, Liao XY, Xue WQ, Chen YB, Zhang Y, Jia WH: HOTAIR, a prognostic factor in esophageal squamous cell carcinoma, inhibits WIF-1 expression and activates Wnt pathway. Cancer Sci 2013;104:1675-1682.
37 Fan J, Zhang Y, Mu J, He X, Shao B, Zhou D, Peng W, Tang J, Jiang Y, Ren G, Xiang T: TET1 exerts its anti-tumor functions via demethylating DACT2 and SFRP2 to antagonize Wnt/beta-catenin signaling pathway in nasopharyngeal carcinoma cells. Clin Epigenetics 2018;10:103.
38 Battistelli C, Sabarese G, Santangelo L, Montaldo C, Gonzalez FJ, Tripodi M, Cicchini C: The lncRNA HOTAIR transcription is controlled by HNF4alpha-induced chromatin topology modulation. Cell Death Differ 2019;26:890-901.
39 Vishnoi K, Mahata S, Tyagi A, Pandey A, Verma G, Jadli M, Singh T, Singh SM, Bharti AC: Human papillomavirus oncoproteins differentially modulate epithelial-mesenchymal transition in 5-FU-resistant cervical cancer cells. Tumour Biol 2016;37:13137-13154.
40 Portari EA, Russomano FB, de Camargo MJ, Machado Gayer CR, da Rocha Guillobel HC, Santos-Reboucas CB, Brito Macedo JM: Immunohistochemical expression of cyclin D1, p16Ink4a, p21WAF1, and Ki-67 correlates with the severity of cervical neoplasia. Int J Gynecol Pathol 2013;32:501-508.
41 Pang T, Li M, Zhang Y, Yong W, Kang H, Yao Y, Hu X: Y Box-Binding Protein 1 Promotes Epithelial-Mesenchymal Transition, Invasion, and Metastasis of Cervical Cancer via Enhancing the Expressions of Snail. Int J Gynecol Cancer 2017;27:1753-1760.
42 Wang N, Wei H, Yin D, Lu Y, Zhang Y, Zhang Q, Ma X, Zhang S: MicroRNA-195 inhibits proliferation of cervical cancer cells by targeting cyclin D1a. Tumour Biol 2016;37:4711-4720.
43 Xu J, Wan X, Chen X, Fang Y, Cheng X, Xie X, Lu W: miR-2861 acts as a tumor suppressor via targeting EGFR/AKT2/CCND1 pathway in cervical cancer induced by human papillomavirus virus 16 E6. Sci Rep 2016;6:28968.
44 Chen YC, Huang RL, Huang YK, Liao YP, Su PH, Wang HC, Chang CC, Lin YW, Yu MH, Chu TY, Lai HC: Methylomics analysis identifies epigenetically silenced genes and implies an activation of beta-catenin signaling in cervical cancer. Int J Cancer 2014;135:117-127.
45 Fu DY, Wang ZM, Li C, Wang BL, Shen ZZ, Huang W, Shao ZM: Sox17, the canonical Wnt antagonist, is epigenetically inactivated by promoter methylation in human breast cancer. Breast Cancer Res Treat 2010;119:601-612.
46 Zhong X, Zhu Y, Mao J, Zhang J, Zheng S: Frequent epigenetic silencing of PCDH10 by methylation in human colorectal cancer. J Cancer Res Clin Oncol 2013;139:485-490.
47 Mizuguchi Y, Saiki Y, Horii A, Fukushige S: Targeted TET oxidase activity through methyl-CpG-binding domain extensively suppresses cancer cell proliferation. Cancer Med 2016;5:2522-2533.
48 Wu H, D'Alessio AC, Ito S, Xia K, Wang Z, Cui K, Zhao K, Sun YE, Zhang Y: Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. Nature 2011;473:389-393.
49 Wu H, D'Alessio AC, Ito S, Wang Z, Cui K, Zhao K, Sun YE, Zhang Y: Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. Genes Dev 2011;25:679-684.