Transcriptional Repression of Tumor Suppressor CDC73, Encoding an RNA Polymerase II Interactor, by Wilms Tumor 1 Protein (WT1) Promotes Cell Proliferation

IMPLICATION FOR CANCER THERAPEUTICS

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Background: Physiological targets of WT1 and the mechanism by which its up-regulation leads to neoplastic transformation remain largely unknown.

Results: WT1 represses expression of tumor suppressor CDC73 and promotes cell proliferation.

Conclusion: Our study elucidates the oncogenic role of WT1, and its up-regulation adds yet another mechanism for CDC73 down-regulation in tumors.

Significance: Targeting WT1 adds novelty to OSCC therapeutics.

The Wilms tumor 1 gene (WT1) can either repress or induce the expression of genes. Inconsistent with its tumor suppressor role, elevated WT1 levels have been observed in leukemia and solid tumors. WT1 has also been suggested to act as an oncogene by inducing the expression of MYC and BCL-2. However, these are only the correlational studies, and no functional study has been performed to date. Consistent with its tumor suppressor role, CDC73 binds to RNA polymerase II as part of a PAF1 transcriptional regulatory complex and causes transcriptional repression of oncogenes MYC and CCND1. It also represses β-catenin-mediated transcription. Based on the reduced level of CDC73 in oral squamous cell carcinoma (OSCC) samples in the absence of loss-of-heterozygosity, promoter methylation, and mutations, we speculated that an inhibitory transcription factor is regulating its expression. The bioinformatics analysis predicted WT1 as an inhibitory transcription factor to regulate the CDC73 level. Our results showed that overexpression of WT1 decreased CDC73 levels and promoted proliferation of OSCC cells. ChIP and EMSA results demonstrated binding of WT1 to the CDC73 promoter. The 5-azacytidine treatment of OSCC cells led to an up-regulation of WT1 with a concomitant down-regulation of CDC73, further suggesting regulation of CDC73 by WT1. Exogenous CDC73 attenuated the protumorigenic activity of WT1 by apoptosis induction. An inverse correlation between expression levels of CDC73 and WT1 was observed in OSCC samples. These observations indicated that WT1 functions as an oncogene by repressing the expression of CDC73 in OSCC. We suggest that targeting WT1 could be a therapeutic strategy for cancer, including OSCC.

The CDC73 (cell division cycle 73; PAF1-RNA polymerase II complex component) gene, also known as HRPT2 (hyperparathyroidism 2), is a tumor suppressor and is mutated in patients with a familial autosomal dominant disorder, hyperparathyroidism-jaw tumor syndrome (1), and in ~70% of all parathyroid carcinomas (2). As part of the PAF1 complex, CDC73 associates with ribonucleic acid (RNA) polymerase II and is involved in several transcriptional and posttranscriptional events (3–6). Studies have shown that overexpression of CDC73 inhibits colony formation and cellular proliferation and induces cell cycle arrest in the G1 phase, indicating its critical role in cell growth and proliferation (7). Immunohistochemistry studies using tissue microarray have shown that CDC73 expression is inversely correlated with tumor size, pathologic stage, and lymphovascular invasion of breast carcinomas (8). Loss of CDC73 expression has been associated with adverse pathological parameters in gastric carcinoma (9). Further, Burton’s tyrosine kinase has been found to increase the abundance of CDC73 in the absence of WNT3A stimulation, and in turn CDC73 acts as a repressor of β-catenin-mediated transcription in human colorectal cancer cells and B cells (10). These findings suggest the potential role of CDC73 as a tumor suppressor gene in malignancies.

Besides mutations, the loss-of-heterozygosity (LOH) and promoter methylation of CDC73 in tumors have been reported as different mechanisms for its down-regulation (11, 12). Recently, a complete loss of CDC73 expression has been reported in parathyroid carcinomas with a single detectable mutation and retention of the wild-type allele in the absence of promoter methylation (13). More recently, we have reported that the up-regulation of oncogenic miR-155 is a major mechanism for the down-regulation of CDC73 in oral squamous cell carcinoma (OSCC) in the absence of LOH, promoter methyla-
tion, and mutation (14). Further, we have shown that miR-155 down-regulates CDC73 by causing its translational repression without affecting its transcript level (14). In addition, we have also identified a subset of OSCC samples having CDC73 down-regulated even at the transcript level in the absence of LOH, promoter methylation, mutation, and miR-155 regulation (14). These results strongly suggest that some other mechanisms, such as mutations in CDC73 intronic regions, alternate epigenetic regulation (e.g. histone modifications), or other regulatory inactivation mechanisms including the concomitant overexpression of an inhibitory transcription factor, may be responsible for CDC73 down-regulation in cancer. Using a combination of bioinformatics and molecular approaches, here we report the identification of an inhibitory transcription factor Wilms tumor protein WT1, encoded by the tumor suppressor gene WT1, which negatively regulates the expression of the tumor suppressor CDC73 via binding its promoter and promotes OSCC cell proliferation.

**MATERIALS AND METHODS**

**Sample Collection**—A total of 24 OSCC samples were ascertained at the Bangalore Institute of Oncology, Bangalore. All OSCC samples were mostly from the tongue and cheek areas of the mouth. This study was performed with informed consent from the patients and approval from the ethics committee of the Bangalore Institute of Oncology. The specimens were obtained as surgical samples from oral cancerous lesions and adjacent normal mucosa (taken from the farthest margin of the surgical resection). The patients had not been treated at the time of surgery. The clinicopathological data for 24 patients are given in supplemental Table S1. Tumors were classified according to TNM (Tumor, Node, and Metastasis) criteria (15).

**Cell Culture, Reporter Assay, Transient Transfection, and Western Blotting**—Cells were grown and maintained in T25 flasks, 6-well, 24-well, or 96-well plates as per requirement. Human cell lines KB (oral squamous cell carcinoma), SCC084...
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(oral squamous cell carcinoma), SCC131 (oral squamous cell carcinoma), and HEK293 (human embryonic kidney) were maintained in DMEM supplemented with 10% fetal bovine serum and 1 × antibiotic/antimycotic solution (all from Sigma-Aldrich) in a humidified chamber with 5% CO2 at 37 °C. HEK293 and KB cells were procured from the National Centre for Cell Science, Pune, India. SCC084 and SCC131 cells were a kind gift from Prof. Susanne M. Gollin, University of Pittsburgh, Pittsburgh, PA.

For the luciferase reporter assay, cells were transfected with an appropriate construct or a combination of constructs using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer’s instruction. The luciferase reporter assay was performed after 24 or 48 h of transfection in SCC131 cells using the Dual Luciferase Reporter Assay system (Promega) as suggested by the manufacturer. The pGLO3-Basic vector was used as a negative control, and the pGLO3-Control harboring a SV-40 promoter was used as a positive control. Cells were also co-transfected with the pRL-TK control vector (Promega), encoding Renilla luciferase, for normalizing transfection efficiency. The results were calculated as the relative luciferase unit of a control over the Renilla luciferase construct. DNA equalization in transfection experiments was carried out using pGLO3-Basic or pcDNA 3.1(+) vectors.

For overexpression studies, SCC131 cells were seeded at a density of 1 × 10⁶ cells/well in 6-well plates. Plasmid constructs were transiently transfected as described above. After 48 h of transfection, cells were used for either the total RNA isolation using the TRIzol reagent (Sigma-Aldrich) or the preparation of total protein lysate using the Celllytic™ Cell Lysis Reagent (Sigma-Aldrich). The Western blot analysis was performed as described by Rather et al. (14). Primary antibodies, anti-CDC73 (product C3620), anti-β-actin, and anti-WT1 (product WH0007490M1-100UG), were purchased from Sigma-Aldrich.

β-Actin was used as a loading control.

Plasmid Constructs—Using bioinformatics analysis, a putative promoter region of CDC73 harboring the WT1 binding site was identified and cloned. Briefly, a reporter construct pGLO3-PDC73 was made by cloning an 825-bp (−425 to +400 bp) long putative promoter region of CDC73 upstream from the luciferase reporter gene in the pGLO3-Basic vector. The promoter region was amplified from normal human genomic DNA using primers PROXF (5′-ATCCCTCGAGAACCACACAACCACAGAGGCCCCAC-3′) and PROHR (5′-TTGAAGCTTCCCTGGGGCCAACGCATGCCG-3′). The forward and reverse primers harbored sites (bases in bold letters) for XhoI and HindIII restriction enzymes, respectively, which allowed directional cloning of the insert. The insert was first cloned in the TA cloning vector pTZFR/T (MBI Fermentas, Burlington, Pittsburgh, PA). From the synthesized stock of cDNA, 1 μl was diluted in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) to a total of 10 μl. Thus, cDNA synthesized from 10 ng of total RNA (1 μl of diluted cDNA) was used as a template for RT-PCR. All real-time PCRs were performed using an ABI Prism 7900 Sequence Detection System (PerkinElmer Life Sciences) and the DyNAmo™ HS SYBR Green qPCR kit (Finnzymes, Espoo, Finland). The thermal cycling conditions included an initial denaturation step at 95 °C for 15 min, 40 cycles at 95 °C for 20 s, 72 °C for 30 s, and 58 °C for 30 s for both GAPDH and CDC73. The relative expression level was computed using the 2−ΔΔCt analysis method and GAPDH or β-actin as an internal control (17). For qRT-PCR analysis of WT1 and CDC73 in SCC131 cells, pcDNA3-GFP was co-transfected with pcDNA 3.1(+)−WT11/− to normalize the transfection efficiency and calculate the relative gene expression. Primers and their conditions used for qRT-PCR analysis of CDC73, β-actin, GFP, and WT1 are available upon request from the authors.

5-Aza-2′ deoxycytidine (5-aza-dC) Treatment of Cell Lines—Three OSCC cell lines (SCC131, SCC084, and KB) were seeded at a density of 1 × 10⁶ cells/90-mm dish. After 24 h, freshly prepared 5-aza-dC (Sigma-Aldrich) was added in the dish to a final concentration of 5 μM. Total RNA was isolated after 3 and 5 days from start of the treatment. Dimethyl sulfoxide (used for dissolving 5-aza-dC)-treated cells were used as a control. The WT1 and CDC73 expression analysis was carried out by qRT-PCR using β-actin as a normalizing control.

Analysis of CDC73 and WT1 Promoter Methylation by Combined Bisulfite Restriction Analysis—Methylation status of the CDC73 and WT1 promoters was examined using the combined bisulfite restriction analysis (COBRA), following a standard procedure of our laboratory as described previously (18). Briefly, 2 μg of genomic DNA in a total volume of 70 μl was denatured with 8 μl of freshly prepared 3 m NaOH by incubating first for 15 min at 37 °C and then for 3 min at 95 °C. DNA was snap-chilled on ice and then treated with 1 ml of bisulfite reagent (3.25 m sodium bisulfite, 2.5 mM hydroquinone, and 0.36 m NaOH) for 16 h at 55 °C in dark. The bisulfite-treated DNA was purified using the Wizard® DNA Clean-Up system (Promega) and eluted in 100 μl of TE buffer, pH 8.0. The eluted DNA was incubated with 3 m NaOH for 15 min at 37 °C fol-
lowed by precipitation with an equal volume of 6 m ammonium acetate and 2 volumes of ethanol at -20 °C overnight. The precipitated DNA was washed with 70% ethanol and dissolved in TE buffer. One µl of this was used in amplification by nested PCR using two sets of primers (outer and inner) specific for the CDC73 promoter region. Primers were designed using the Methprimer database. Amplions from the outer and inner sets of primers were of 517 and 351 bp, respectively, and the amplions from the inner primer set harbored a total of 9 Acil restriction enzyme sites. To test the efficiency of Acil restriction enzyme, the 507-bp ASPM amplicon from an unconverted DNA was used as a positive control. Approximately 500 ng of DNA was digested with Acil at 37 °C for 5 h. The digests were run on a 2% agarose gel and visualized by ethidium bromide staining. The restriction site of Acil is as follows: 5’-CCGC-3’. If the C residue in the CpG dinucleotide is methylated, it will remain C following the bisulfite treatment, resulting in the digestion of the amplicon by Acil. However, if it is not methylated, it will convert to U (T in the PCR product), resulting in the loss of Acil restriction site and undigestion of the amplicon. Therefore, digestion of the amplicon is the readout of promoter methylation.

The WT1 promoter sequence analyzed for methylation was the same as described previously (19). Sodium bisulfite-treated DNA was again amplified by nested PCR using two sets of primers (outer and inner) specific for the WT1 promoter region. The final amplicon of 315 bp, generated by the inner set of primers, was digested with Acil. The 433-bp WRAP73 amplicon from an unconverted DNA with 2 Acil sites was used as a positive control. Bisulfite-converted DNA from a normal individual was used as a negative control for the WT1 promoter methylation analysis. Primer sequences used in the promoter methylation analysis are available upon request from the authors.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation (ChIP) was performed using HEK293 cells and an anti-WT1 antibody (product WH0007490M1-100UG) from Sigma-Aldrich, as described previously (20). To determine whether the CDC73 promoter was pulled down, a 266-bp region of the promoter from -4 to +262 bp harboring the binding site for WT1 was amplified using qPCR. The -fold enrichment was calculated using 2^ΔΔCt method: ΔCt = Ct of the ChIP DNA − Ct of the input DNA, and ΔΔCt = ΔCt of sample (immunoprecipitated DNA of CDC73 or MYC) − ΔCt of IgG control (SABiosciences). Primers used to amplify the promoter regions of CDC73 and MYC (used as a positive control) are available upon request from the authors.

Electrophoretic Mobility Shift Assay—In addition to the ChIP assay, binding of WT1 to the CDC73 promoter was also analyzed by electrophoretic mobility shift assay (EMSA) using a standard laboratory procedure. Briefly, end-labeled double-stranded oligonucleotide probes for the EMSA were prepared using T4 polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol; BRIT, Hyderabad, India). The DNA-protein binding was carried out in a 25-µl reaction using 4 µg of HEK293 nuclear extract in a binding buffer containing 20 mM HEPES, pH 7.5, 60 mM KCl, 0.2 mM EDTA, 10% glycerol, 1 mM DTT, and 1 x protease inhibition mixture (all from Sigma-Aldrich). The nuclear extract from HEK293 cells was mixed with all of the components except an appropriate end-labeled probe and incubated for 10 min. For competition experiments, a 50-fold excess of the same unlabeled probe was added 15 min prior to the addition of the end-labeled probe. To analyze the super-shift, an anti-WT1 antibody (Sigma-Aldrich, St. Louis, MO) was added 15 min prior to the addition of an end-labeled probe. Subsequently, 50,000 CPM of an end-labeled probe was added and incubated for 20 min. All incubation steps were performed on ice. Following incubation, DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel in 0.5× TBE buffer at 4 °C and 50 V. The WT1 binding to a specific binding site in the MYC promoter has been demonstrated previously (21) and was therefore used as a positive control for the validation of WT1 binding to the CDC73 promoter. Oligonucleotide sequences for the CDC73 promoter used in EMSA are as follows: 5’-GGCGGGGGGAGGGGAAGATGGCGG-GGA-3’ and 5’-GGGTCCGCCCATCTTCCCCCCCTCGCCT-3’, whereas oligonucleotide sequences for the MYC (c-MYC) promoter used in EMSA are as follows: 5’-AAAGCAG-GAGGCGTGGGGGAAAAA-3’ and 5’-TTTTCCCCACGG-CCCTCTGTTTT-3’ (21). A double-stranded probe was made by mixing equal quantity of two oligonucleotides in TE, pH 8.0, followed by heating at 65 °C for 10 min. The mixture was then slowly cooled to room temperature for 30 min. The WT1 binding sites are bifolded.

LOH Analysis at the CDC73 Locus—For LOH studies, total genomic DNA from blood and OSCC samples was isolated using the Wizard® Genomic DNA purification kit (Promega) according to the manufacturer’s instruction. Matched normal and OSCC DNA samples were genotyped using four highly polymorphic microsatellite markers as described by Rather et al. (14).

Mutation Analysis—The entire coding region of CDC73 including its exon/intron junctions was amplified using gene-specific primers and sequenced on an ABIprism A310-automated sequencer (Invitrogen). Primer sequences used in the mutation analysis are available upon request from the authors.

Cell Proliferation Assay—The rate of cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (14).

Apoptosis Assay—The rate of apoptosis was measured using the CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (BioVision, Milpitas, CA) as described previously (14).

Statistical Analysis—An independent two-tailed Student’s t test was performed to determine the significance of difference between two experiments.

RESULTS

In Silico Identification of WT1 as a CDC73 Regulator—To predict the transcription factors that may regulate CDC73 expression, we took a consensus approach using two bioinformatics tools, the transcription regulatory element database and the transcription start site database, to retrieve the CDC73 promoter. Both the tools identified the same region of 1000 bp from nucleotide positions -600 to +400 as the putative CDC73 promoter (Fig. 1A). To functionally characterize the CDC73 promoter, a construct pGL3-PCDC73 harboring an 825-bp

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Statistical Analysis—An independent two-tailed Student’s t test was performed to determine the significance of difference between two experiments.
promoter region from −425 to +400 bp was generated (Fig. 1B), transfected in SCC131 cells, and the Dual Luciferase Reporter assay was performed. The results showed that the region has a significantly higher promoter activity over the pGL3-Basic vector (Fig. 1C). The CDC73 promoter sequence was then used as a query sequence in the MatInspector Professional program database which identified putative binding sites for several transcription factors, including that of an inhibitory transcription factor WT1 (Fig. 1A). The ClustalW alignment showed that the WT1 binding site in the CDC73 promoter is conserved in different species (Fig. 1D), underscoring that it may have a basal role to play in the regulation of CDC73 expression.

WT1 Overexpression Knocks Down CDC73 Expression—To investigate the role of WT1 in the regulation of CDC73 expression, the pcDNA3.1(+)-WT1/+/- construct, encoding for the major isoform of WT1 (WT1 (+KTS) +17AA) with a strong transcriptional repressor activity (22), was transiently co-expressed in SCC131 cells in increasing amounts with 1 μg of pcDNA3-GFP followed by qRT-PCR analysis to check for the expression of WT1 and CDC73. The results showed that, as expected, WT1 down-regulated CDC73 in a dose-dependent manner (Fig. 2A). Regulation of CDC73 by WT1 was further investigated by the luciferase reporter assay. Transient co-transfection of pGL3-PCDC73 (luciferase reporter construct driven by the CDC73 promoter region) with the WT1 construct pcDNA3.1(+)-WT1/+/- in SCC131 cells down-regulated the luciferase reporter activity in a dose-dependent manner (Fig. 2B). When we co-transfected the pGL3-PCDC73(mut) construct harboring the mutated WT1 binding site with pcDNA3.1(+)-WT1/+/- in SCC131 cells, the luciferase reporter activity was restored to the normal value (Fig. 2B), suggesting that WT1 regulates CDC73 expression directly in a site-specific manner.

5-Aza-dC Treatment of Cells Up-regulates WT1 and Down-regulates CDC73—Methylation of the WT1 promoter has been reported previously (19). To validate further that WT1 regulates CDC73 expression, we speculated that if the WT1 promoter is also methylated in our panel of cell lines, then the treatment of these cell lines with 5-aza-dC, a DNA methyl
WT1 knockdown induced the expression of MYC control cells without affecting the expression of a nonspecific target. Body pulldown chromatin with expression via binding its promoter, we performed Promoter-specific enrichment of the antibody pulldown chromatin with ChIP and EMSA. The ChIP results showed amplification and beled probe from the showed that preincubation of nuclear extract with an end-label transferase inhibitor, should result in WT1 overexpression with a concomitant down-regulation of CDC73. Therefore, we treated a panel of cell lines (KB, SCC084, and SCC131) with 5-aza-dC and dimethyl sulfoxide (vehicle control). Total RNA was isolated from cells after 3 and 5 days of the treatment, and the qRT-PCR analysis was performed to assess the levels of WT1 and CDC73. As expected, the WT1 expression was upregulated with a concomitant down-regulation of CDC73 in SCC131 cells (Fig. 2C). KB and SCC084 cells did not show changes in the levels of either of the genes following the 5-aza-dC treatment (data not shown). To validate if WT1 overexpression was indeed due to its promoter methylation, we analyzed its promoter methylation by COBRA. The results showed methylation of its promoter in SCC131 cells only (Fig. 2D). These results further underscore that WT1 represses the promoter activity of CDC73.

WT1 Down-regulates CDC73 Expression via Binding Its Promoter—To determine whether WT1 down-regulates CDC73 expression via binding its promoter, we performed ChIP and EMSA. The ChIP results showed amplification and specific enrichment of the antibody pulldown chromatin with CDC73 promoter-specific primers, suggesting binding of WT1 to the promoter in vivo (Fig. 2E). As expected, the ChIP results also showed amplification and specific enrichment of the antibody pulldown chromatin with MYC promoter-specific primers used as a positive control (Fig. 2E). The results of EMSA showed that preincubation of nuclear extract with an end-labeled probe from the CDC73 promoter and an anti-WT1 antibody caused supershift (Fig. 2F, lane 3), confirming the binding of WT1 to the CDC73 promoter. These results suggested that WT1 is exercising its oncogenic role by down-regulation of tumor suppressor CDC73. The results also showed binding of WT1 to the MYC promoter as reported previously (Fig. 2F, lane 8).

Knockdown of WT1 Induces CDC73 Expression—To recapitulate our finding that high expression of CDC73 was observed due to the promoter methylation of its inhibitory transcription factor WT1 in SCC131 cells, the siRNA-mediated knockdown of WT1 was performed in HEK293 cells, and its effect on CDC73 expression was analyzed. Interestingly and as expected, WT1 knockdown induced the expression of CDC73 in HEK293 cells without affecting the expression of a nonspecific target control ESRRA (estrogen-related receptor α). However, control cells transfected with siRNA targeting IGFBP2 (insulin-like growth factor-binding protein 2) did not affect WT1 or CDC73 expression compared with mock (Fig. 3). Thus, our results suggest that CDC73 is a direct and specific target of WT1.

WT1 Regulates OSCC Cell Proliferation by Targeting CDC73—To determine whether the WT1-mediated reduction of CDC73 has a functional relevance in cell growth and proliferation, we knocked down CDC73 expression by transfecting the WT1 construct pcDNA3.1(+)-WT1+/− in SCC131 cells and quantitated cell proliferation by the 3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide assay. As expected, the results demonstrated that the reduced CDC37 level was associated with an increased cell proliferation (Fig. 4, left and middle panels). Because WT1 can target several genes having tumor suppressor function, we assessed the tumorigenic potential of WT1 by targeting CDC73. The CDC73 construct pcDNA3-HA-CDC73 was co-transfected with the WT1 construct pcDNA3.1(+)-WT1+/− in SCC131 cells, and the rate of proliferation was measured. The results showed the restoration of cell proliferation compared with cells transfected with the control vector pcDNA3-HA (Fig. 4, middle panel).

Recently, we have shown that CDC73 reduces cell proliferation by apoptosis induction (14). Therefore, we also measured the rate of apoptosis in SCC131 cells transfected with the empty vector pcDNA3-HA, pcDNA3.1(+)-WT1+/−, and pcDNA3-HA-CDC73 separately or pcDNA3.1(+)-WT1+/− and pcDNA3-HA-CDC73 together (Fig. 4, right panel). As expected, overexpression of CDC73 significantly increased the rate of apoptosis compared with cells transfected with pcDNA3-HA (Fig. 4, right panel). Because the WT1 overexpression induces the rate of cell proliferation (Fig. 4, middle panel), the restoration of exogenous CDC73 expression by co-transfecting cells with pcDNA3.1(+)-WT1+/− and pcDNA3-HA-CDC73 led to an increased rate of apoptosis further (Fig. 4, right panel), suggesting that CDC73 attenuated the pro-oncogenic effect of WT1 in SCC131 cells by apoptosis induction. These results suggested that WT1 functions in OSCC as an oncogene, in part by targeting CDC73.

Correlation between WT1 and CDC73 Expression Levels in OSCC Samples—After validating CDC73 as the target of WT1 by bioinformatics and in vitro assays, we quantitated the expression levels of WT1 and CDC73 by qRT-PCR in 24 OSCC and their matched normal oral tissue samples (Fig. 5). The results showed an inverse correlation between the expression levels of WT1 and CDC73 in 13 of 24 OSCC samples. For example, 10 of 24 OSCC samples (samples 153, 189, 199, 211, 151, 207, 213, 154, 194, and 202) showed a significant up-regulation of WT1 with a concomitant significant down-regulation of CDC73 compared with their matched normal oral tissues (Fig. 5 and supplemental Table S1). However, 3 of 24 OSCC samples (samples 172, 173, and 152) showed a significant down-regulation of WT1 with a concomitant significant up-regulation of WT1 by targeting CDC73. The CDC73 construct pcDNA3-HA-CDC73 was co-transfected with the WT1 construct pcDNA3.1(+)-WT1+/− in SCC131 cells, and the rate of proliferation was measured. The results showed the restoration of cell proliferation compared with cells transfected with the control vector pcDNA3-HA (Fig. 4, middle panel).
CDC73 compared with their matched normal oral tissues (Fig. 5 and supplemental Table S1). To exclude the possibility of alternate mechanisms as the cause of CDC73 down-regulation in OSCC, we selected 9 OSCC samples (samples 153, 189, 211, 151, 207, 213, 154, 194, and 202) with a down-regulated level of CDC73 and analyzed for LOH at the CDC73 locus and promoter methylation. Further, 5 of these 9 OSCC samples (samples 189, 211, 207, 213, and 202) were also analyzed for muta-
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DISCUSSION

CDC73 is a key player in regulation of the WNT-β-catenin signaling pathway (10), which is reported to be dysregulated in OSCC (23). Further, the mechanism of its complete loss in a subset of parathyroid tumors in the absence of null mutations and promoter methylation remains to be elucidated (13). We and others have recently shown that Bruton’s tyrosine kinase and mir-155 play an important role in the posttranscriptional regulation of CDC73 (10, 14). However, the reports regarding its transcriptional regulation are lacking. For the first time, here we have shown that WT1 transcriptionally represses CDC73 expression via binding its promoter.

Due to the presence of zinc fingers in its C-terminal half, WT1 has been found to be a potent transcriptional regulator of genes important for cellular growth and metabolism, including extracellular matrix components, growth factors, and other transcription factors (24). Despite the fact that it was first categorized as a tumor suppressor gene, experiments have suggested that WT1 also functions as an oncogene on the basis of the following findings. The first is overexpression of the wild-type WT1 gene in both leukemias and solid tumors (25–28), including OSCC (29). The second is growth suppression of leukemic and solid tumor cells by treatment with WT1 antisense oligomers (30). The third is inhibition of differentiation, but induction of proliferation of wild-type WT1 gene-transfected myeloid progenitor cells in response to granulocyte colony-stimulating factor and a stronger capacity of the cells with high levels of WT1 to develop into leukemias (31). The fourth is a phase II clinical study showing that WT1 vaccination could induce WT1-specific cytotoxic T lymphocytes and cancer regression without damage to normal tissues (32, 33). Finally, reports suggest that WT1 can act as an oncogene, depending on the type of cell line by inducing the expression of proto-oncogenes MYC and BCL-2 (21, 34). According to our knowledge, these are only the correlational studies, and no functional study has been carried out to date. Therefore, the complete mechanism regarding the oncogenic role of WT1 remains unknown. Being an inhibitory transcription factor, it will be interesting to see whether WT1 causes cancer by targeting tumor suppressor genes. As observed during the present study, the tumor suppressor gene CDC73 is one of them. To date, there are no reports of an increased risk of oral cancer in Wilms tumor patients. However, Mikami et al. (29) have recently shown that WT1 is up-regulated in OSCC. Therefore, OSCC can serve as an excellent model system to gain insight into the functional role of WT1 with a pro-oncogenic characteristic in different cancers.

Our findings of a differential level of WT1 in various cell lines (data not shown) and a subset of paired normal oral and OSCC tissue samples (Fig. 5) support that the WT1-mediated control of CDC73 is operational in cells that actively produce it. Further, the expression analysis of WT1 showed that it is highly enriched in a majority of OSCC samples compared with their normal counterparts and had an inverse effect on CDC73 expression, suggesting its key role in the pathogenesis of OSCC by targeting CDC73 (Figs. 4 and 5). Overexpression of WT1 down-regulated CDC73 both at the transcript and the protein levels in SCC131 cells (Figs. 2A and 4) and reversed the effect of CDC73 by promoting cell proliferation (Fig. 4, middle panel). Further, the combined overexpression of CDC73 and WT1 in SCC131 cells abrogated the oncogenic potential of WT1 by apoptosis induction (Fig. 4, right panel). We have shown earlier that overexpression of CDC73 decreases the proliferation of OSCC cells, whereas the knockdown of CDC73 expression reverses its effect by inducing OSCC cell proliferation (14).

In summary, our study has shown for the first time that WT1 binds to the promoter of a tumor suppressor gene CDC73, negatively regulates its activity, and promotes proliferation of OSCC cells. Further, the present study not only addresses a previously undescribed role of WT1 in OSCC as an oncogene, but also suggests that the restoration of CDC73 level by the use of an inhibitor to WT1 could be a prolific strategy to treat cancers, including OSCC.

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tions in CDC73. The results showed that these OSCC samples did not show LOH, promoter methylation, or any mutation (supplemental Table S1), again validating the fact that CDC73 is a biological target of oncogenic WT1, and the transcriptional repression of CDC73 by WT1 is a major mechanism for CDC73 down-regulation in OSCC. Further, we have analyzed the correlation between CDC73 or WT1 transcript levels and clinical parameters using Fisher’s exact t test and did not observe any correlation between their expression levels and clinical parameters, such as patient gender, patient age, tumor stage, and tumor site (data not shown).
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