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TCF7L1 recruits CtBP and HDAC1 to repress *DICKKOPF4* gene expression in human colorectal cancer cells

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ABSTRACT

The T-cell factor/Lymphoid enhancer factor (TCF/LEF; hereafter TCF) family of transcription factors are critical regulators of colorectal cancer (CRC) cell growth. Of the four TCF family members, TCF7L1 functions predominantly as a repressor of gene expression. Few studies have addressed the role of TCF7L1 in CRC and only a handful of target genes regulated by this repressor are known. By silencing *TCF7L1* expression in HCT116 cells, we show that it promotes cell proliferation and tumorigenesis *in vivo* by driving cell cycle progression. Microarray analysis of transcripts differentially expressed in control and TCF7L1-silenced CRC cells identified genes that control cell cycle kinetics and cancer pathways. Among these, expression of the Wnt antagonist DICKKOPF4 (*DKK4*) was upregulated when TCF7L1 levels were reduced. We found that TCF7L1 recruits the C-terminal binding protein (CtBP) and histone deacetylase 1 (HDAC1) to the *DKK4* promoter to repress *DKK4* gene expression. In the absence of TCF7L1, TCF7L2 and β-catenin occupancy at the *DKK4* promoter is stimulated and *DKK4* expression is increased. These findings uncover a critical role for TCF7L1 in repressing *DKK4* gene expression to promote the oncogenic potential of CRCs.

1. Introduction

The Wnt/β-catenin signaling pathway controls cellular proliferation within the intestinal crypt microenvironment [1] and mutations in components of this pathway are highly prevalent in spontaneously arising CRCs [2,3]. These mutations drive tumorigenesis, in part, by deregulating expression of direct Wnt/β-catenin target genes, which are controlled by T-cell factor (TCF) transcription factors [3,4]. The four TCF family members, TCF7, LEF1, TCF7L1 and TCF7L2 are expressed in the intestinal epithelium and in CRCs [5,6]. Whereas TCF7, LEF1, and TCF7L2 have been thoroughly evaluated in established human CRC cell lines, the function of TCF7L1 in these cells has received little attention and few target genes directly regulated by TCF7L1 have been described.

TCF family members contain a conserved DNA binding domain that mediates their association with Wnt-responsive DNA regulatory elements (WREs) [7]. However, upon binding WREs, TCF family members differentially affect target gene expression [7]. TCF7 and LEF1 function as transcriptional activators, whereas TCF7L2 can activate or repress gene expression depending on cellular context [7,8]. TCF7L1 predominantly functions as a repressor of Wnt target gene expression [7]. The *Xenopus* homolog of human TCF7L1, xTCF3, recruits the C-terminal binding domain protein one (CtBP1) to repress target gene expression [9]. CtBP1 interacts with histone deacetylases (HDACs), to repress transcription at target genes [10]. While TCF7L2 has been shown to recruit CtBP1 to repress target gene expression in human CRC cells [11], whether TCF7L1 operates using a similar mechanism has not been reported.

Recent work has indicated an oncogenic role for *TCF7L1* in human malignancies, including breast cancer, leukemia, and basal cell carcinomas [12–14]. In CRC, recurrent chromosomal translocations were found that fused *TCF7L1* with *NAV2*, although the potential

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role of the putative NAV2-TCF7L1 chimeric protein in tumorigenesis was not addressed [2]. Recently, Murphy et al. reported that TCF7L1 promotes CRC growth and identified EPH receptor B3 (*EPHB3*) as a direct target gene [15]. However, this study did not address the mechanism by which TCF7L1 represses *EPHB3* gene expression. Whether additional target genes are directly regulated by TCF7L1 in CRC and whether TCF7L1 recruits corepressor complexes to repress transcription are areas of open investigation.

In this report, we confirm prior findings demonstrating that TCF7L1 promotes HCT116 cellular proliferation, progression through the cell cycle, and tumorigenesis *in vivo* [15]. We identify the secreted Wnt antagonist, *DICKKOPF-4* (*DKK4)* as a direct TCF7L1 target gene and show that TCF7L1 represses its expression by recruiting a CtBP/HDAC1 complex to its proximal promoter. In TCF7L1-silenced cells, a TCF7L2/β-catenin complex occupies the *DKK4* promoter to increase *DKK4* expression. These findings support the conclusion that TCF7L1 serves an oncogenic role in CRCs, in part, through repressing expression of the Wnt antagonist DKK4.

2. Materials and methods

2.1. Cell culture

HCT116 (ATCC) and HEK293FT (Invitrogen) cell lines were maintained at 37 °C in 5% CO_2 in DMEM supplemented with 10% Fetal Bovine Serum (Atlantic Biologicals), 50 units/ml penicillin (Corning Mediatech), 2 mM Glutamax (ThermoFisher Scientific), and 0.1 mg/ml streptomycin (Corning Mediatech). HEK293FT cells were supplemented with 500 μg/ml G418 (VWR).

2.2. Lentiviral shRNAs

Lentiviral shRNA constructs that target *TCF7L1* were obtained from Open Biosystems (GE Healthcare). The sequences and clone numbers are listed in Table S1. The control shRNA plasmid containing a scrambled shRNA sequence was previously described [16]. Lentiviral particles were produced by transfecting HEK293FT cells with 3 μg of each packaging plasmid (pLP/VSVG, pLP1, and pLP2) and 3 μg of the respective lentiviral shRNA construct using Lipofectamine 2000 (Life Technologies). Media containing virus was harvested at 24 and 48 h following transfection and cleared of cell debris by centrifugation at 1500 × g for 5 min at room temperature. The media was supplemented with 6 μg/ml hexadimethrine bromide (Sigma) and added to HCT116 cells for 24 h. Media was replaced with fresh DMEM and cells were harvested for assays 72 h later.

2.3. Transcript analysis by reverse transcription and real time PCR (RT-qPCR)

Total RNA was isolated from approximately 5 × 10^6 cells using TRIZol reagent (Invitrogen) and cDNAs were synthesized as previously described [16]. Transcripts were measured as previously described using the primers listed in Table S1 [17]. Transcript abundance was normalized to *GAPDH* and presented as relative expression using the $2^{-\Delta\Delta Ct}$ equation. For HDAC inhibitor studies, cells were treated with 400 nM trichostatin A (TSA) (Sigma) for 24 h prior to analysis.

2.4. Western blot

Protein extracts were prepared from approximately 5 x 10^6 cells and analyzed by western blot as described previously [18]. Blots were probed with primary antibodies directed against TCF7L1 (Cell Signaling Technologies, 2883, 1:1000 dilution) and α-Tubulin (Sigma, T9026, 1:1000 dilution).

2.5. Cellular proliferation

Approximately 7.5 × 10^4 control and TCF7L1-silenced cells were seeded in triplicate in a 6-well dish. On the indicated day post-seeding, cells were stained with 0.2% trypan blue, and counted with a Cellometer cell counting chamber (Nexcelom Bioscience).

2.6. Cell cycle analysis

Approximately 1.5 × 10^6 control and TCF7L1-silenced cells were seeded in a 100-mm culture dish. The following day, cells were harvested by trypsinization and centrifugation at 500 × g, for 5 min at room temperature. Cells were treated with 1 ml of propidium iodide staining solution for 30 min at 4 °C and analyzed by flow cytometry as previously described [19]. The percentage of cells in each phase of the cell cycle was calculated using ModFit LT software (Verity Software House).

2.7. Mouse xenograft assays

Approximately 5 × 10^6 control and TCF7L1-silenced cells were resuspended in 150 μl of 1× PBS and injected subcutaneously into the flanks of athymic nude mice (Charles River Labs, strain #088). Beginning on day 13 post-injection, tumors were measured every two days. At day 21, tumors were removed and weighed. The Pennsylvania State University College of Medicine Institutional Care and Use Committee (IACUC) approved all animal protocols used in this study.

2.8. Microarray analysis

Total RNA was isolated from control and TCF7L1-silenced cells using the RNeasy Mini Kit (Qiagen). cRNA was synthesized using the TotalPrep Amplification Kit (Ambion) according to manufacturer's instructions. Microarray analysis was performed using the Illumina HT-12 Beadchip (Illumina), which was scanned with a BeadArray Reader (Illumina). The scanned data was analyzed using GenomeStudio 1.0 (Illumina) and results were exported to GeneSpring Gx11 (Agilent Technologies). Initial quality control (positive and negative controls), background subtraction, and intra-array normalization were performed. Changes in gene expression upon *TCF7L1* silencing were defined by two-class significance analysis of microarray data (SAM) of three samples in each class with a *P*-value less than 0.05 using a 1.4-fold threshold. The microarray data is available at the gene expression omnibus (GEO) data repository (#GSE92446).

2.9. Luciferase reporter assays

An 819-base pair region of the *DKK4* promoter [20] was amplified from HCT116 genomic DNA in a standard PCR reaction [16]. The DNA oligonucleotides used in the reaction are listed in Table S1. The PCR product was subcloned as an NheI/KpnI fragment into the pGL3 basic plasmid (Promega). This plasmid is referred to as pGL3-*DKK4*p. Luciferase assays were conducted as previously described [21]. For *TCF7L1* knockdown experiments, 24 h after transduction with control or TCF7L1-specific shRNAs, HCT116 cells were seeded in quadruplicate in a 24-well plate. The following day, cells were transfected with 100 ng of pGL3-*DKK4*p or empty vector, 2 ng of pLRL-SV40-*Renilla* luciferase plasmid (Promega, E2231), and pBluescript to obtain a final DNA concentration of 1 μg. For *TCF7L1* overexpression experiments, HCT116 cells were transfected as described above with 100 ng or 200 ng of pCMV-*TCF7L1* plasmid

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