The β-Catenin/TCF-4 Complex Imposes a Crypt Progenitor Phenotype on Colorectal Cancer Cells

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Abstract

The transactivation of TCF target genes induced by Wnt pathway mutations constitutes the primary transforming event in colorectal cancer (CRC). We show that disruption of β-catenin/TCF-4 activity in CRC cells induces a rapid G1 arrest and blocks a genetic program that is physiologically active in the proliferative compartment of colon crypts. Coincidently, an intestinal differentiation program is induced. Thus, the β-catenin/TCF-4 complex constitutes the master switch that controls proliferation versus differentiation in healthy and malignant intestinal epithelial cells.

Introduction

The colorectal mucosa contains large numbers of invaginations termed the crypts of Lieberkühn. Epithelial renewal occurs in these crypts through a coordinated series of events involving proliferation, differentiation, and migration towards the intestinal lumen. Pluripotent stem cells at the crypt bottom generate progenitors that occupy the lower third of the crypt. Cells in this amplification compartment divide approximately every 12 hr. In the midcrypt region, the cells differentiate into one of the functional cell types of the colon. At the epithelial surface, cells undergo apoptosis and/or extrusion into the lumen. The entire process takes approximately 3–5 days[1].

The transition of an intestinal epithelial cell into a fully transformed, metastatic cancer cell requires mutations in multiple proto-oncogenes and tumor suppressor genes[2]. The APC gene, originally cloned from the rare genetic disorder familial adenomatous polyposis, is mutated in most sporadic CRCs. The APC protein resides in the destruction complex, together with GSK3β, axin/conductin, and β-catenin. In this complex, phosphorylation by GSK3β targets β-catenin for ubiquitination and destruction by the proteasome. Wnt signaling inhibits GSK3β activity. Then, β-catenin accumulates in the nucleus, where it binds members of the TCF family and converts these WNT effectors from transcriptional repressors into activators[3]. In cancer, truncating mutations in APC and axin/conductin, as well as mutations in the GSK3β-target residues in β-catenin, all lead to the formation of constitutive nuclear β-catenin/TCF complexes[4-8]. Activating mutations of the WNT pathway are the only known genetic alterations in early premalignant lesions in the intestine, such as aberrant crypt foci and small polyps[9]. Thus, these mutations may initiate the transformation of colorectal epithelial cells.

In the intestinal epithelium, Tcf-4 is the most prominently expressed TCF family member[4]. Gene disruption has revealed that Tcf-4 is required to establish the proliferative progenitors of the prospective crypts in the embryonic small intestine[10]. A second TCF family member, TCF-1, is expressed in the intestinal epithelium predominantly as a dominant-negative isoform, which lacks the N-terminal β-catenin interaction domain. Genetic evidence suggests that Tcf-1 acts as an antagonist of Tcf-4 in the formation of polyps in an APC^{min} background[11].

To understand the contribution of constitutive β-catenin/TCF-4 activity to the colorectal transformation process, we have undertaken a large-scale analysis of the downstream genetic program activated by β-catenin/TCF in CRC cells. We have subsequently analyzed the expression and activities of individual β-catenin/TCF-4-regulated genes in a physiological context. Our results indicate that the β-catenin/TCF-4 complex inhibits differentiation and imposes a crypt progenitor-like phenotype on CRC cells.

Results

Inhibition of β-catenin/TCF in CRC cells results in cell cycle arrest

To determine the role of β-catenin/TCF complexes in CRC, we constructed cell lines carrying doxycycline-inducible expression plasmids encoding the β-catenin binding N terminus of TCF-4 fused to a nuclear localization signal (NLS) (N-TCF4). As the recipient CRC cell lines, we chose Ls174T, which expresses mutant β-catenin protein, yet is diploid and carries wild-type alleles of p53 and APC. Strong nuclear dnTCF-4 staining was observed after 4–8 hr of doxycycline (Dox) induction (Fig. 1A). CRC cell lines that carry WNT pathway mutations constitutively activate TCF reporters (pTOPFlash)[4]. We selected several clones in which the expression of N-TCF4 completely abrogated this constitutive pTOPFlash activity (Fig. 1B). The results of 2 independent clones are shown.

Upon induction of N-TCF4 by doxycycline in the LS174T the CRC cells underwent a robust G1 arrest as shown by FACS scatter analysis (Fig. 2A). This block in cell proliferation was also visualized by crystal violet staining (Fig. 2B). No apoptosis was noted (not shown). The results were identical with the induction of dnTCF-4 and dnTCF-1 abrogated β-catenin/TCF activity on the cell cycle of Ls174T and DLD1 cells[12].

In conclusion, the inhibition of β-catenin/TCF signaling in CRC cells results in a quick and robust cell cycle arrest.

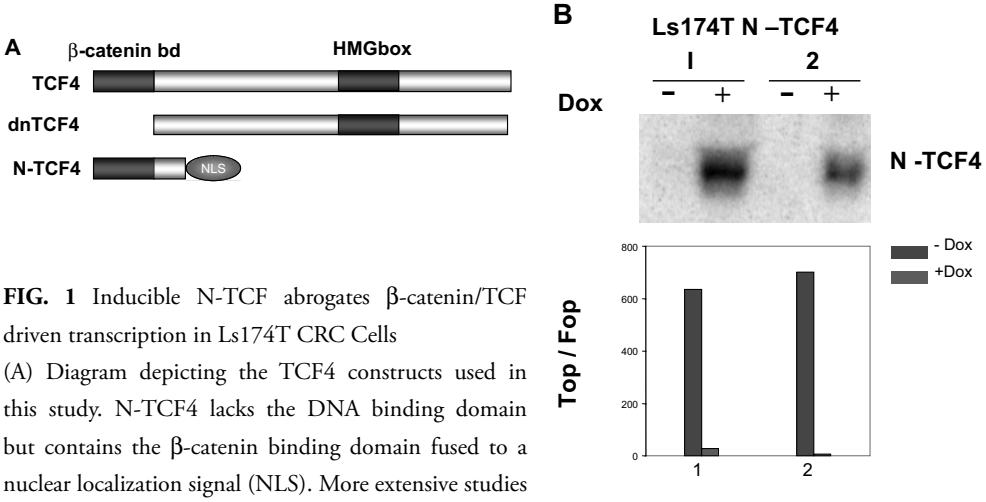

FIG. 1 Inducible N-TCF abrogates β-catenin/TCF driven transcription in Ls174T CRC Cells

(A) Diagram depicting the TCF4 constructs used in this study. N-TCF4 lacks the DNA binding domain but contains the β-catenin binding domain fused to a nuclear localization signal (NLS). More extensive studies have been carried out with the dnTCF4 (12).

(B) N-TCF4 is induced as early as 4 hr after induction with doxycycline as analyzed by Western blot in two independent LS174T cell lines. The induction of N-TCF4 abrogates β-catenin/TCF driven transcription in two independent Ls174T clones. Activity of the TCF-reporter TOPFlash (purple bars) and control FOPFlash (green bars) after 24 hr with or without doxycycline (dox) is shown. Renilla luciferase levels were used as transfection controls.

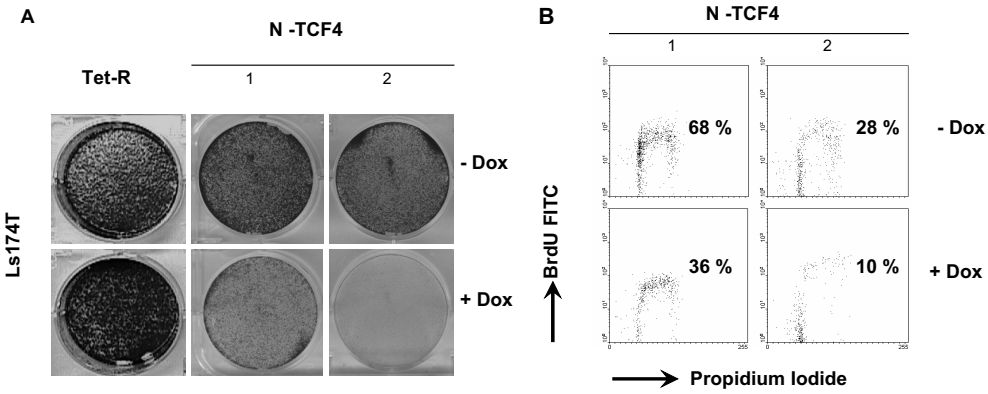

FIG. 2 Inhibition of TCF/β-catenin-driven transactivation by induction of NTCF-4 results in cell cycle arrest

Ls174T show a dramatic reduction in S phase cells upon NTCF-4 expression.

(A) Proliferation was halted in Ls174T transfectants. This was visualized by crystal violet staining of cell cultures after 5 days of N-TCF expression.

(B) The scatter profile of cells after 20 hr with or without doxycycline is shown. Numbers refer to the percentage of cells in S phase for each cell line analyzed. The results are representative of several independent experiments.

The genetic program driven by β-catenin/TCF in CRC cells

Previous studies have identified individual target genes of TCFs in various cellular systems. By DNA array analysis on the induction of dnTcf-4 in Ls174T and DLD1 cells it has been shown which genes were regulated by the induction of dnTCF-4 in Ls174T CRC cells[12]. This analysis defined a small set of 120 downregulated and 115 increased genes[12]. Remarkbly, many of the increased entries represented differentiation markers of mucosecretory and/or absorptive intestinal cells. These data were confirmed by Northern blot analysis. This study concluded that the inhibition of β-catenin/TCF-4 activity induced cell cycle arrest and differentiation[12]. We investigated whether in the 2 independent N-TCF4 LS174T cell lines the same set of genes were up- c.q. downregulated upon induction with doxycyclin. This analysis has been carried out by Northern Blot analysis (Fig. 3). Indeed, abrogation of β-catenin/TCF activity induced the mRNA expression of classical differentiation markers such as mucin2 (MUC2), fatty acid binding protein 2 (FABPL), carbonic anhydrase II (CAII), and $p21^{CIP1/WAF1}$ mRNAs after 24 hours of N-TCF4 induction with doxycycline. Moreover, abrogation of β-catenin/TCF activity downregulated BMP-4, c-Myc, EphB3 and NRP/B. These are target genes of the Wnt pathway normally expressed in the proliferating region of the crypt.

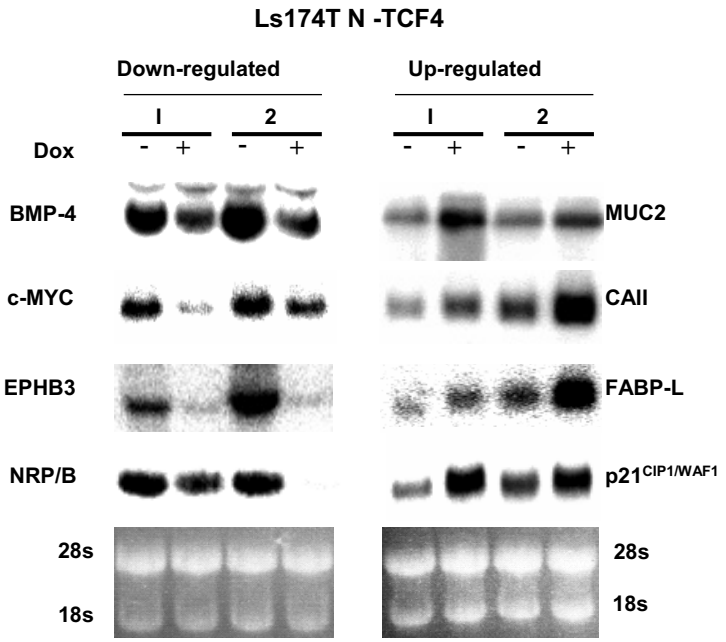

FIG. 3 Representative examples of several genes regulated upon 24 hours of induction with doxycyclin in Ls174T.

The bottom panel shows the 28S ribosomal RNA as a loading control. Abrogation of β-catenin/TCF activity induced the mRNA expression of classical differentiation markers such as mucin2 (MUC2), fatty acid binding protein 2 (FABPL), carbonic anhydrase II (CAII), and p21CIP1/WAF1 mRNAs after 24 hours of N-TCF4 induction with doxycycline. Abrogation of β-catenin/TCF activity downregulated BMP-4, c-Myc, EphB3 and NRP/B expression. These are target genes of the Wnt pathway normally expressed in the proliferating region of the crypt

In conclusion, the inhibition of the deregulate Wnt signaling pathway in CRC by the induction of NTCF-4 leads to the induction of genes known to be expressed in differentiated cells and the downregulation of genes known to be expressed in proliferating cells.

The genetic program controlled by β-catenin/TCF in CRC cells is physiologically active in colonic epithelium

To show that the genetic program controlled by β-catenin/TCF in CRC cells is physiologically active in colonic epithelium we performed Immunohistochemical analyses on early neoplastic lesions. A strict correlation between nuclear β-catenin (Fig. 4A) and the expression of target genes such as EPHB2 (Fig. 4B) was observed in early colorectal lesions. Many other downregulated genes listed were overexpressed in early intestinal polyps from Min

mice or in aberrant crypt foci (ACF) from FAP patients[12]. More strikingly, all tested target genes were expressed also in the proliferative compartment of normal colon crypts (e.g. EPHB2 and[12]). A complementary domain of expression was observed for genes strongly upregulated upon induction of NTCF-4 in Ls174T cells. One such example is carbonic anhydrase II, which was restricted to the top of the crypts and the surface epithelium (Fig. 4F) but was absent from polyp cells arising in this area (Fig. 4E).

In conclusion, the genetic program controlled by β-catenin/TCF in CRC cells is indeed physiologically active in normal adult colonic epithelium

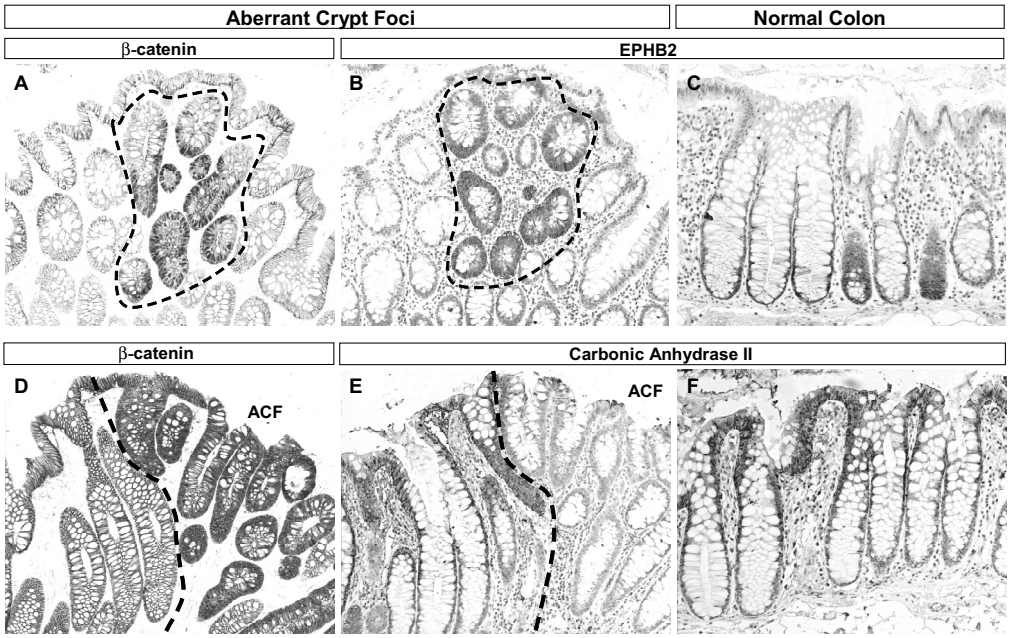

FIG. 4. Genes downstream of β-catenin/TCF activity show a complementary expression pattern to intestinal markers of differentiation

Representative examples of the inverse expression domain of downregulated (B and C) or upregulated (E and F) genes in our system, in normal colon (C and F) or early colorectal lesions (B and E).(A and B) The expression of nuclear β-catenin (A) perfectly correlates with that of EPHB2 tyrosine kinase receptor (B) in aberrant crypt foci (ACF). Stainings were performed on serial sections of early human lesions. The dashed lines delimit the same ACF in both stainings.(C and F) EPHB2 and CAII show a complementary domain of expression in healthy tissue. EPHB2 is expressed at the bottom of the crypts (C), while CAII is only present in cells at the top of the crypts and surface epithelium of the colon (F).(D and E) ACFs that colonize the surface epithelium show strong cytoplasmic and nuclear β-catenin (D) and are completely negative for carbonic anhydrase II (CAII) (E). The dashed line indicates the boundary between normal tissue and the ACF areas in serial sections.

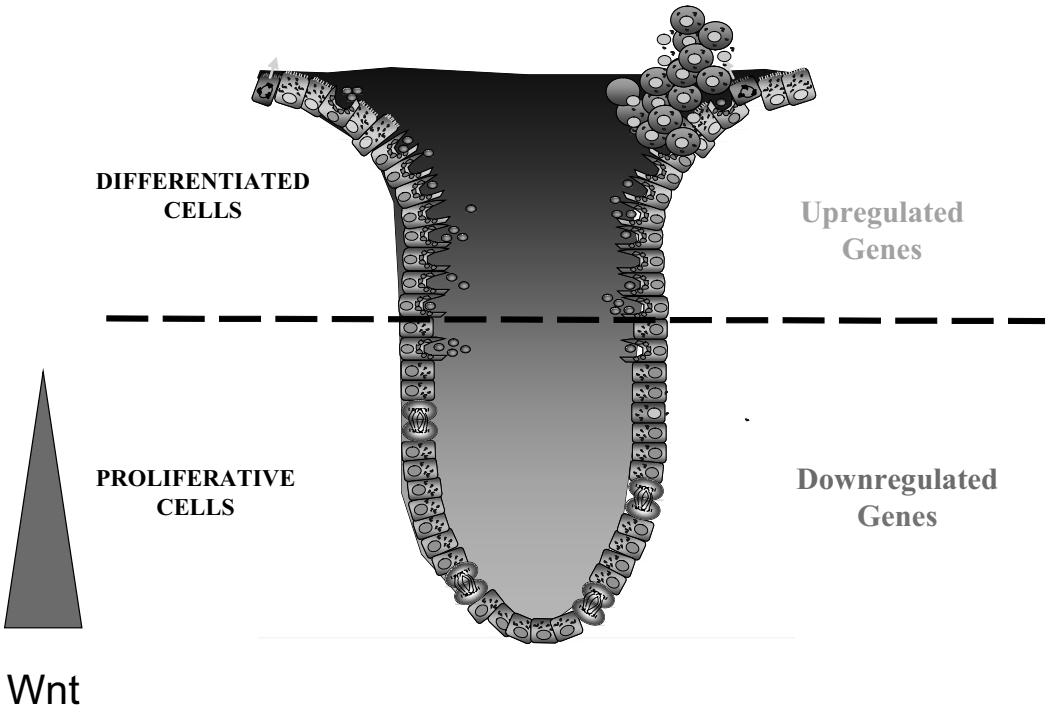

FIG. 5. Schematic representation of a colon crypt and proposed model for polyp formation.
At the bottom third of the crypt, the progenitor proliferating cells accumulate nuclear β-catenin. Consequently, they express β-catenin/TCF target genes. As the cells reach the midcrypt region, β-catenin/TCF activity is downregulated and this results in cell cycle arrest and differentiation. Cells undergoing mutation in APC or β-catenin become independent of the physiological signals controlling β-catenin/TCF activity. As a consequence, they continue to behave as crypt progenitor cells in the surface epithelium, giving rise to tumors.

Concluding remarks

These observations imply that the Wnt signaling pathway constitutes the dominant switch between the proliferating progenitor and the differentiated intestinal cell. This is still recapitulated in the CRC cells used in this study, despite the presence of multiple additional mutations in these cells. These data lead to a model for the maintenance of colon and for polyp formation. At the bottom third of the crypt, the progenitor proliferating cells accumulate nuclear β-catenin as a result of Wnt signaling. Consequently, they express β-catenin/TCF target genes. As the cells reach the midcrypt region, β-catenin/TCF activity is downregulated and this results in cell cycle arrest and differentiation. Cells undergoing mutation in APC or β-catenin become independent of the physiological signals controlling β-catenin/TCF activity. As a consequence,

they continue to behave as crypt progenitor cells in the surface epithelium, giving rise to tumour formation. The current study validates the disruption of the β-catenin/TCF complex as a therapeutic strategy to revert the transformed phenotype in colorectal cancer.

Experimental Procedures

Cell Culture and Transfections

T-REx system (Invitrogen) was used according to manufacturer's instructions to generate N-TCF4 inducible CRC cell lines. Resistant colonies were tested for induction by immunocytochemical staining.

Cell Cycle Analysis

3×10^6 Ls174T cells were seeded in 9 cm dishes and doxycycline was added (1 μg/ml). After 20 hr, BrdU (Roche) was added for 20 min. Cells were then fixed in ethanol 70%. Nuclei were isolated and incubated with α-BrdU-FITC (BD), and cell cycle profiles were determined by FACS analysis. Crystal violet staining on methanol-fixed cells was done on cells after 5 days induction.

Immunohistochemistry

Antibodies used: EPHB from R&D systems; carbonic anydrase II from Rockland; and β-catenin from Transduction Laboratories. Immunostainings were performed according to standard procedures. Envision+ kit (DAKO) was used as a secondary reagent. Stainings were developed using DAB (brown precipitate). Slides were counterstained with hematoxylin.

Reporter Assays

Transient transfections of the appropriate Firefly luciferase reporters and Renilla luciferase as a transfection control were performed with Fugene 6 (Roche) and measured with the Dual Luciferase Reporter Assay System (Promega).

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