Cdx2 Controls Expression of the Protocadherin Mucdhl, an Inhibitor of Growth and β-Catenin Activity in Colon Cancer Cells

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BACKGROUND & AIMS: The intestine-specific homeobox transcription factor Cdx2 is an important determinant of intestinal identity in the embryonic endoderm and regulates the balance between proliferation and differentiation in the adult intestinal epithelium. Human colon tumors often lose Cdx2 expression, and heterozygous inactivation of Cdx2 in mice increases colon tumorigenesis. We sought to identify Cdx2 target genes to determine how it contributes to intestinal homeostasis. **METHODS:** We used expression profiling analysis to identify genes that are regulated by Cdx2 in colon cancer cells lines. Regulation and function of a potential target gene were further investigated using various cell assays. **RESULTS:** In colon cancer cell lines, Cdx2 directly regulated the transcription of the gene that encodes the protocadherin Mucdhl. Mucdhl localized to the apex of differentiated cells in the intestinal epithelium, and its expression was reduced in most human colon tumors. Overexpression of Mucdhl inhibited low-density proliferation of colon cancer cells and reduced tumor formation in nude mice. One isoform of Mucdhl interacted with β-catenin and inhibited its transcriptional activity. **CONCLUSIONS: The transcription factor Cdx2 activates expression of the protocadherin Mucdhl, which interacts with β-catenin and regulates activities of intestinal cells. Loss of Cdx2 expression in colon cancer cells might reduce expression of Mucdhl and thereby lead to tumor formation.**

Keywords: Tumor Suppressor; cdhr5; Colorectal Cancer; CRC.

Despite its complex architecture, the intestinal epithelium represents the most vigorously renewing adult tissue in mammals.[1] Thus, dynamic temporal and spatial coordination of various cellular processes is not only required during fetal development of the gut but also throughout adult life to maintain homeostasis.

Alterations of the signaling pathways that ensure controlled renewal of the gut are frequently found in gut pathologies, notably colorectal cancer,[2] which corresponds to the second cause of cancer-related death in the Western world.[3] This is well illustrated by the prominent loss of function of APC, which in particular regulates the protein levels of β-catenin: in addition to its adhesive function at the membrane, stabilized β-catenin relays in the nucleus the activity of the Wnt pathway, which has a central role in the control of proliferation, cell stemness, differentiation, and migration in the gut.[4,5]

Another molecule required for the homeostasis of the gut and whose expression is frequently reduced in colon tumors[6] is the homeodomain transcription factor Cdx2. Cdx2 specifies cell fate or axial position at different developmental stages,[7] but a series of recent studies confirmed that Cdx2 is a major player in both the developing and the mature intestinal epithelium. Cdx2 expression in the embryo per se is detected around embryonic day (E) 8.5 and becomes progressively restricted to the intestinal epithelium at the adult stage.[8] Endoderm-specific Cdx2 ablation from E9.5 onward results in colon agenesis, with the ectopic activation of a foregut/esophageal differentiation program reflecting an anterior homeotic transformation.[9] Intestine-specific Cdx2 ablation around E14 is also lethal and leads to abnormal morphogenesis of the small intestine, with shortened villi and poorly differentiated, nonpolarized enterocytes.[10] Strikingly, even intestine-specific loss of Cdx2 at the adult stage led to chronic diarrhea and death, with small intestine dilation and immature enterocytes, indicating that Cdx2 is required to maintain gut homeostasis throughout life.[11] Accordingly, reduction of Cdx2 expression promotes tumor progression in animal models of colon cancer,[12,13] whereas forced expression of Cdx2 in colon cancer cells decreases metastasis in nude mice.[14]

Altogether, these data correlate quite well with experiments performed in vitro. Indeed, forced expression of Cdx2 in intestinal cell lines enhances intestinal differentiation[15,16] and adhesion[17] while decreasing proliferation.[14,18] On the opposite, silencing of Cdx2 disrupts apicobasal polarity[10] and enhances motility.[14] On the molecular level, a certain number of genes regulated by Cdx2 have been identified, namely intestine-specific genes involved in the digestive function.[8] As direct targets mediating the other functions of Cdx2 remain more elusive, we took advantage of microarray-based studies performed with intestinal cells showing modification of Cdx2 expression. One of the genes whose expression paralleled the one of Cdx2 was Cdhr5, also called Mucin and cadherin like

Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; PLA, proximal ligation assay; qPCR, quantitative polymerase chain reaction; RT, reverse transcription.

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(Mucdhl). This gene encodes several splicing isoforms of a protocadherin mostly expressed in the kidney, liver, and gut.[19–21] Protocadherins represent a group of less conserved members of the Cadherin superfamily that are membranous glycoproteins mediating Ca^{2+}-dependent cell-to-cell adhesion. All Mucdhl isoforms exhibit 4 cadherin extracellular motifs but differ by the presence of several mucin-like tandem repeats located downstream of the cadherin motifs, a transmembrane domain, and an intracellular domain containing many potential protein-binding motifs.[20,21] In this study, we investigated the regulation of Mucdhl expression in intestinal cells and initiated a comparative analysis of the function and mode of action of Mucdhl isoforms.

Materials and Methods

Small Interfering RNA

Stealth/small interfering RNA (siRNA) duplex oligoribonucleotides were from Invitrogen (Carlsbad, CA): CDX2HSS141544 (siRNA@Cdx2 544), CDX2HSS141546 (siRNA@Cdx2 546), and medium GC Duplex number 2 as negative control.

Mucdhl-Expressing Cell Lines

The T-REx System (Invitrogen) was used to establish control and Mucdhl-inducible cell lines. First, HCT116TR cells, which constitutively express the tetracycline repressor, were established by transfection of HCT116 cells with pcDNA6/TR and blasticidin selection. Clone 7 of HCT116TR was transfected with pcDNA4/TO, pcDNA4/TO-DHLM/Flag or pcDNA4/TO-DHLL/HA and submitted to Zeocin selection. Several independent stable clones were obtained that expressed Flag-tagged Mucdhl M or HA-tagged Mucdhl L. These clones were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, Blasticidin (4 μg/mL; Invitrogen), Zeocin (100 μg/mL; Invitrogen), and antibiotics. Induction of expression was performed by adding doxycycline (1 μg/mL; Sigma, St. Louis, MO) to the medium.

Wound Healing and Grafting

Wound healing assay with Caco2/TC7 cells and grafting of HT29 cells were previously described.[14,22] Subcutaneous injections of HCT116 Mucdhl stable cells (1.10^6 cells/each side) in female nu/nu mice (Elevage Janvier, France) were performed similarly, with half of the mice receiving doxycycline (400 μg/mL) in their drinking water.[14,22] Mice were killed (clone M, 8 weeks; clone L, 4 weeks), and tumors measured.

Transfections

Plasmid transfections were performed with Lipofectamine 2000 (Invitrogen) or Jetprime (Polyplus Transfection, Illkirch, France), except for Luciferase assays in which JetPEI (Polyplus Transfection) was used. siRNA duplexes were transfected with Lipofectamine RNAiMAX (Invitrogen).

RNA Extraction and Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated using TRI Reagent (MRC, Cincinnati, OH). Semiquantitative reverse-transcription polymerase chain reactions (RT-PCR) were performed as described[14] with the following primer pairs: hMucdhl L (forward: CTC CCA CCA ACC AAC CAC; reverse: CAT ATC CAC CAC CGA GAA GC), hMucdhl M (forward: TGG AGG GAG AGG TTG TGC T; reverse: GGC CGC CAC CTG TGG AGG), hCdx2 (forward: AAG CCG AGC TAG CCG CCA CGC TGG; reverse: TGA GAG CCA GGT CTG TAG GTC TAT) and as internal control hTBP (forward: CGT GTG AAG ATA ACC CAA G; reverse: ATT GGA CTA AAG ATA GGG A).

Luciferase Assays

Luciferase assays were performed with the Dual-Luciferase Reporter Assay (Promega, Madison, WI) as described.[14] Data presented correspond to a representative experiment performed in triplicate.

In Situ Proximal Ligation Assay

Cells seeded in 8-well CultureSlides (BD Falcon, Mountain View, CA) were transfected and treated overnight with MG132 (10 μmol/L; Sigma) before fixation (Paraformaldehyde 4%, 15 minutes) and permeabilization (Triton X-100 0.5% in phosphate-buffered saline, 30 minutes). Slides were processed for in situ proximal ligation assay (PLA) with the Duolink II Detection Reagents Green, Duolink II PLA probe anti-Mouse PLUS, and Duolink II PLA probe anti-Rabbit Minus (Olink Bioscience, Uppsala, Sweden) as recommended. Primary antibodies were rabbit anti-β-catenin (clone 6B3, 1/150; Cell Signaling Technology, Danvers, MA) and mouse anti-Flag (clone M2, 1/500; Sigma). To check the specificity, control experiments omitting one of the primary antibodies were performed. Imaging was based on optical sectioning using the ApoTome system (Zeiss, Oberkochen, Germany).

Colony Formation and Soft Agar Assays

For colony formation assays, 250 cells in 1 mL of medium were added to 1 mL of medium without or with doxycycline (2 μg/mL) per well of a 6-well plate (3 replicates) and allowed to grow for 14 days before fixation (70% ethanol), staining with Crystal violet (0.05% in phosphate-buffered saline), and counting of the colonies.

For soft agar assays, cells were mixed with Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 0.4% low melting point agarose (Promega); 1.5 mL of this solution (5000 cells) were added to each well of a 6-well plate previously coated with 1.5 mL of 0.6% low melting point agarose (3 replicates). After 30 minutes, 1 mL of growth medium without or with doxycycline (1 μg/mL) was added per well. Medium was changed every 2–3 days. After 3 weeks, colonies were fixed, stained, and counted.

Statistical Analysis

$^*P < .01$ and $^{**}P < .001$ given by a 1-way analysis of variance test followed by a Newman–Keuls test. Additional materials and methods are presented in Supplementary Materials and Methods.

Results

Expression of Mucdhl Depends on the Presence of Cdx2 in Intestinal Cells

We previously reported similar changes in messenger RNA expression and/or protein distribution during in vitro wound healing and tumor dissemination in colon cancer for several molecules. In particular, β-catenin accumulated in the nucleus of migrating cells at the wound edge or at the invasive front of tumors, whereas Cdx2 expression was down-regulated in both situations.[14] To