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TNF-α-induced down-regulation of CDX2 suppresses *MEP1A* expression in colitis

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ABSTRACT

Background/aims: High levels of pro-inflammatory cytokines are linked to inflammatory bowel disease (IBD). The transcription factor *Caudal*-related homeobox transcription factor 2 (CDX2) plays a crucial role in differentiation of intestinal epithelium and regulates IBD-susceptibility genes, including *meprin 1A (MEP1A)*. The aim was to investigate the expression of CDX2 and MEP1A in colitis; to assess if they are regulated by tumor necrosis factor-α (TNF-α), and finally to reveal if CDX2 is involved in a TNF-α-induced down-regulation of *MEP1A*. Methods: Expression of CDX2 and MEP1A was investigated in colonic biopsies of ulcerative colitis (UC) patients and in dextran sodium sulfate (DSS)-induced colitis. CDX2 protein expression was investigated by immunoblotting and immunohistochemical procedures. *CDX2* and *MEP1A* regulation was examined in TNF-α-treated Caco-2 cells by reverse transcription-polymerase chain reaction and with reporter gene assays, and the effect of anti-TNF-α treatment was assessed using infliximab. Finally, *in vivo* CDX2–DNA interactions were investigated by chromatin immunoprecipitation. Results: The CDX2 and MEP1A mRNA expression was significantly decreased in active UC patients and in DSS-colitis. Colonic biopsy specimens from active UC showed markedly decreased CDX2 staining. TNF-α treatment diminished the CDX2 and MEP1A mRNA levels, a decrease which, was counteracted by infliximab treatment. Reporter gene assays showed significantly reduced *CDX2* and *MEP1A* activity upon TNF-α stimulation. Finally, TNF-α impaired the ability of CDX2 to interact and activate its own, as well as the *MEP1A* expression. Conclusions: The present results indicate that a TNF-α-mediated down-regulation of CDX2 can be related to suppressed expression of *MEP1A* during intestinal inflammation.
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1. Introduction

Inflammatory bowel disease (IBD), of which ulcerative colitis (UC) and Crohn's disease (CD) are the two main entities, is characterized by an abnormal immune response to antigens of the intestinal luminal content leading to a persistent inflammatory state. Clinical symptoms of IBD include abdominal pain, diarrhea, rectal bleeding, and weight loss [1]. The etiology of IBD is unknown, but involves a complex interplay of environmental factors including the gut microbiota, as well as genetics affecting the intestinal barrier function. Thus, the intestinal barrier is pivotal for the maintenance of intestinal homeostasis, and a linkage between the key signaling pathways involved in inflammation and regulators of homeostasis is often observed [2].

One of the key factors involved in maintaining the intestinal epithelium homeostasis is the *Caudal*-related homeobox transcription factor 2 (CDX2). CDX2 is an intestine-specific transcription factor, which is essential for the regulation of genes related to epithelial functions [3–8]. A conditional knock-out of the murine *Cdx2* gene results in a disorganization in the differentiation of the intestinal epithelium, as the colonocytes differentiate into a gastric rather than into an intestinal phenotype [9]. Therefore, CDX2 is a crucial regulator of intestinal epithelial function, controlling the balance between differentiation and proliferation of intestinal epithelial cells (IECs) [10]. Given the importance of CDX2 in regulating numerous intestinal genes on the one hand, and preserving intestinal homeostasis on the other hand, it

Abbreviations: CD, Crohn's disease; CDX2, *Caudal*-related homeobox transcription factor 2; ChIP, chromatin immunoprecipitation; CK20, cytokeratin 20; CLDN2, claudin-2; DAI, disease activity index; DSS, dextran sodium sulfate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK3β, glycogen synthase kinase-3β; HA, hemagglutinin; HNF4α, hepatocyte nuclear factor 4 alpha; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IFX, infliximab; IHC, immunohistochemistry; INF-γ, interferon-γ; MEP1A, meprin 1A; MUC2, mucin 2; NF, nuclear factor; PEPT1, peptide transporter 1; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; RPLP0, ribosomal protein large p0; TNF-α, tumor necrosis factor-α; UC, ulcerative colitis

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is obvious that a proper regulation of *CDX2* is pivotal for maintaining an unbreached intestinal mucosa. In fact, several studies have reported that *Hepatocyte nuclear factor 4 alpha* (*HNF4α*), *Meprin 1A* (*MEP1A*), *Peptide transporter 1* (*PEPT1*), *Claudin-2* (*CLDN2*), and *Mucin 2* (*MUC2*) are susceptible genes associated with IBD [11–15] and experimental colitis [16–18]. Interestingly, all these genes are CDX2-targets [19–22].

Until now only limited efforts have been allocated into the investigation of CDX2 in relation to intestinal inflammation. In one recent study, a diminished CDX2 expression was revealed in UC [23]. On the contrary, another study did not find any correlation between CDX2 positive cells and the degree of inflammation in UC [24]. Thus, the source of CDX2 expression in the inflamed mucosa of IBD patients remains to be investigated. Further, we have earlier demonstrated that *MEP1A*, a membrane-associated protein that is expressed by IECs and hydrolyzes a variety of peptide and protein substrates [25,26], is a direct target gene of CDX2 [19], and recently a down-regulation of MEP1A in UC has been reported [12]. Additionally, *Cdx2*$^{+/-}$ mice or mice lacking *Mep1a* developed more severe inflammation than wild-type mice in response to dextran sodium sulfate (DSS)-induced colitis [12,17,27].

To date, the regulation of CDX2 in mucosal inflammation is rather unclear, except that one study has reported that CDX2 expression is repressed by tumor necrosis factor-α (TNF-α) signaling [28]. TNF-α is a pro-inflammatory cytokine, found at increased concentrations in the inflamed intestines of UC and CD [29,30], and neutralizing the biological activity of TNF-α with infliximab (IFX), a chimeric monoclonal antibody against human TNF-α, may induce clinical remission in IBD [31]. Thus we speculated that an increased amount of TNF-α in inflamed intestinal mucosa might suppress *MEP1A* gene expression at the transcriptional level by inhibiting CDX2 regulation. To address this question, we aimed to investigate the correlation between TNF-α and the expression of CDX2 and MEP1A in intestinal inflammation. We analyzed the influence of TNF-α on the expression and activity of *CDX2* and *MEP1A* in inflammation, and further studied the effect of anti-TNF-α treatment with IFX using *in vitro* experiments.

2. Material and methods

2.1. Patients and tissue samples

Twenty-two patients underwent a routine colonoscopy due to their clinical condition and were included into the study: six UC patients with active disease and eight with quiescent disease, together with eight healthy controls (i.e. patients undergoing an endoscopy due to gastrointestinal symptoms but where all clinical and paraclinical investigations subsequently turned out to be normal). The disease activity of all UC patients were before the colonoscopy graded in accordance with Mayo score [32]: a score ≤2 as the disease in remission, and >2 (max. 12) as active stages of the disease. Patient characteristics are shown in Table 1. No pregnant or breastfeeding women, individuals with other inflammatory diseases, an anamnesis of cancer or psychiatric disorders were included, and none had an infection within the last 2 weeks. No selection was made for age, gender, or disease duration. None of the included patients had received IFX or recent (within 14 days) use of antibiotics. Six biopsies each of approximately 15 mg each were obtained from the descending colon in each patient during the colonoscopy.

Table 1
Patient characteristics.

	Controls	Quiescent UC	Active UC
n	8	8	6
Gender (male/female)	5/3	1/7	3/3
Age—years, median (IQR)	52 (43–60)	54 (44–58)	42 (37–50)
Years with disease (<10 yrs/>10 yrs)	–	6/2	4/2

UC, ulcerative colitis; IQR, interquartile range.

The study was approved by the Scientific Ethics Committee of the Capital Region of Denmark. All patients gave their informed consent to participate in the study.

2.2. Cell culture and treatment

The human colonic Caco-2 cells (American Tissue Type Culture Collection; Rockville, MD, USA) were maintained and grown as previously described [33]. Caco-2 cells were seeded in 24-well plates (NUNC Brand, Thermo Fisher, Rochester, NY, USA) and grown to >95% confluence. Cells were then stimulated in medium with or without interferon-γ (IFN-γ) (10 nM; Sigma-Aldrich, St. Louis, MO, USA) or TNF-α (10 nM; R&D Systems, Minneapolis, MN, USA) in the presence or absence of IFX (100 μg/ml; Centocor Ortho Biotech Inc., Horsham, PA, USA). The cells were harvested after 24 h.

2.3. RNA extraction and quantitative RT-PCR

Total RNA from isolated colonocytes from tissue samples were isolated and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed as previously described [34]. Target gene expressions were calculated by their ratios to the housekeeping reference gene human *Ribosomal Protein Large P0* (*RPLP0*) or mouse *β-actin*, which were amplified in parallel reactions. Sequences of the primers are listed in Supplementary Table S1. The cycling conditions are available upon request.

2.4. Protein extraction and immunoblotting

Colonocytes were isolated as earlier described in detail [35]. Primary colonocytes were lysed with RP1 lysis buffer (Macherey-Nagel, Düren, Germany) and protein extracts were obtained as earlier described in detail [34]. The primary antibodies were: CDX2 (1:1000; mouse monoclonal; BioGenex Laboratories Inc., Fremont, CA, USA), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:20,000; mouse monoclonal; Fitzgerald, Concord, MA, USA).

2.5. Transient transfection, treatment and luciferase assays

For transient transfection of promoter constructs, Caco-2 cells were seeded in 24-well plates at a density of 5×10^4 cells/well and transiently transfected the following day using ExGen500 *in vitro* transfection reagent (Fermentas, St. Leon-Rot, Germany). In treatment experiments, the medium was changed 24 h after transfection, washed, and subsequently stimulated with or without TNF-α (10 nM) for the next 24 h. The constructs containing the human *CDX2* or *MEP1A* promoter have earlier been described [19]. A glycogen synthase kinase-3β (GSK3β) promoter construct (pGL4-GSK3β) containing the region −1173 to −128 was used as a non-TNF-α responsive promoter. For co-transfections, full-length CDX2 (a CDX2 expressing plasmid), CDX2Δ55–136 (an expression vector with a deleted CDX2 activation domain between amino acids 55 and 136); both kindly provided by Dr. John P. Lynch [36], empty pcDNA3.1 (+) expression vector, or a combination of these expression vectors were added together with 0.1 μg pCMV-lacZ (as internal transfection control) and normalized to a total amount of 0.3 μg DNA/well with pBluescript SK$^+$ plasmid. Forty-eight hours post-transfection, cells were harvested and lysed, and luciferase and β-galactosidase activities were determined using the Dual Light system (Perkin Elmer, Waltham, MA, USA) according to manufacturer's instructions. Each luciferase activity was normalized to β-galactosidase activity. Reporter gene activities of both treated and untreated samples were normalized to the activity of the promoterless pGL4-Basic vector and presented as fold induction.

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