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Regulation of Laminin γ2 expression by CDX2 in colonic epithelial cells is impaired during active inflammation†

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Abstract

The expression of *Caudal*-related homeobox transcription factor 2 (CDX2) is impaired by tumor necrosis factor-α (TNF-α)-mediated activation of nuclear factor-κB (NF-κB) in ulcerative colitis (UC). Laminin subunit γ2 (LAMC2) is an epithelial basement membrane protein implicated in cell migration, proliferation, differentiation as well as tumor invasion and intestinal inflammation, and its expression is enhanced by TNF-α in a NF-κB-dependent regulation of the recently identified LAMC2 enhancer. The aim was to determine whether CDX2 is involved in the basal regulation of LAMC2 in epithelial cells and to assess the influence of inflammation. Transcriptional regulation of LAMC2 was examined by reporter gene assays, overexpression, and shRNA-mediated knock-down of CDX2. CDX2-DNA interactions were assessed by chromatin immunoprecipitation on Caco-2 cells without or with TNF-α as well as in purified colonic human epithelial cells. Immunohistochemical staining and quantitative reverse-transcription polymerase chain reaction analyses were used to measure the expression of CDX2 and LAMC2 in colonic biopsies from healthy controls and patients with UC. These data indicate that CDX2 directly regulates LAMC2 gene expression through interaction with elements in the LAMC2 promoter region. We further revealed an inverse effect of inflammation on CDX2 and LAMC2. The data presented provide a novel insight into how CDX2 is implicated in the transcriptional regulation of LAMC2 in intestinal epithelial cells, a function that is impaired during mucosal inflammation where a high level of TNF-α is present. This article is protected by copyright. All rights reserved.
Introduction

Inflammatory bowel disease (IBD) is a chronic disease of as yet unknown etiology, comprised of two main subtypes – ulcerative colitis (UC) (Ordas et al., 2012) and Crohn’s disease (Baumgart and Sandborn, 2012). IBD is of multifactorial origin and involves a complex interaction between genetic, luminal, and environmental factors that trigger an abnormal mucosal immune response to bacterial antigens that harms the mucosal barrier (Atreya and Neurath, 2015; Cammarota et al., 2015; de Souza and Fiocchi, 2016; Kostic et al., 2014). In particular, the intestinal epithelium has a strategic position as a protective physical barrier to luminal microbiota (Coskun, 2014a; Koch and Nusrat, 2012), and intestinal barrier dysfunction therefore has a key pathogenic role in IBD (Maloy and Powrie, 2011). Indeed, several IBD-susceptibility genes implicated in mucosal barrier function have been found (Jostins et al., 2012; McCole, 2014; Mokry et al., 2014), and it is revealed that the expression of genes associated with intestinal permeability is restored in patients with UC responding to biologic therapy (Toedter et al., 2012). The epithelium is constituted by a single-cell layer of specialized intestinal epithelial cells (IECs) that are essential to maintain intestinal homeostasis. Hence, an intact differentiation system for correct IEC formation has a fundamental role in intestinal homeostasis, and several crucial genes for the differentiation of IECs have been demonstrated to become aberrantly expressed in UC (Ahn et al., 2008; Coskun et al., 2012; Coskun et al., 2014; Zheng et al., 2011).

One important transcription factor controlling the balance between cell proliferation and differentiation in intestinal epithelium is the Caudal-related homeobox transcription factor 2 (CDX2). CDX2 is an intestine-specific transcription factor, which is crucial for the regulation of genes related to epithelial functions, and has an essential role in intestinal homeostasis and inflammation (Coskun et al., 2011; Coskun, 2014b). CDX2 is down-regulated both in the inflamed epithelium of patients with UC and in vitro by pro-inflammatory tumor necrosis factor (TNF-α) (Coskun et al., 2012; Kim et al., 2002). Moreover, it has been demonstrated that the regulatory effect of TNF-α on CDX2 is mediated by nuclear factor-κB (NF-κB) activation (Coskun et al., 2014). Importantly, CDX2 regulates genes in the Wnt/β-catenin degradation complex to keep cell differentiation and proliferation in balance (Olsen et al., 2013). However, an increased infiltration of inflammatory cells expressing TNF-α induces proliferation by suppressing epithelial CDX2 expression and as a consequence increases the risk of generating tumor-initiating cells (Schwitalla et al., 2013).
Laminins are a family of large heterotrimeric glycoproteins which are essential components of various basement membranes. The intestinal basement membrane is a specialized extracellular matrix component that separates epithelial cells from the underlying stromal layer and this membrane is in addition to laminins mainly composed of type IV collagens, nidogens, and proteoglycans.

The pathogenesis of several human disorders have been associated with defects in assembly or composition of the basement membrane (Yurchenco and Patton, 2009). The laminin trimers are comprised of three non-identical chains classified as α, β, and γ. In mammals, more than fifteen laminin trimers formed by different subunit composition of five α-, three β-, and three γ-chains have been reported. Several αβγ heterotrimeric complexes of laminins exist in the human intestine, including laminin-332 (composed of α3β3γ2 subunits), which show developmental and position specific expression along the crypt-villus axis (Benoit et al., 2012; Teller and Beaulieu, 2001). Among the laminins, laminin-332 is well associated with cutaneous epithelial cell functions, including formation of hemidesmosomes and maintenance of skin integrity (Rousselle and Beck, 2013). Ablation of laminin γ2 in mice (encoded by the Lamm2 gene), unique to the laminin-332 isoform, leads to a skin blistering phenotype and perinatal death due to malnutrition (Meng et al., 2003). The human ortholog, LAMC2 is important for cell migration and is overexpressed in several types of human cancers. Thus, LAMC2 is considered a molecular marker of invading cancer cells, and its expression correlates with a poorer prognosis of patients with cancer (reviewed in ref. (Garg et al., 2014)).

Altered expression profiles of several laminin subunits have been reported in patients with IBD and experimental colitis (Bjerrum et al., 2014; Bouatrouss et al., 2000; Koutroubakis et al., 2003; Schmehl et al., 2000; Spenle et al., 2014; Toedter et al., 2012). Moreover, genomic loci linked to the LAMB1 gene has specifically been found to strongly associate with UC-susceptibility (Thompson and Lees, 2011). Taken together, these studies highlight the importance of a controlled expression of laminins in intestinal homeostasis and indicate that dysregulated laminins may contribute to the pathogenesis of UC (Spenle et al., 2012).

LAMC2 is regulated in vitro by several pathways, including Wnt/β-catenin signaling (Hlubek et al., 2001; Olsen et al., 2000; Salo et al., 1999) and stimulated by pro-inflammatory cytokines including TNF-α (Boyd et al., 2014; Francoeur et al., 2004). However, little is known about the transcriptional regulation of LAMC2 in intestinal inflammation (Simon-Assmann et al., 2010). Of note, the TNF-α-mediated increase of
endogenous LAMC2 expression is dependent on NF-κB activation, and moreover, TNF-α regulates LAMC2 transcriptional activity by an NF-κB-bound enhancer (Boyd et al., 2014).

In the present study, we provide data that implicate CDX2 as an important factor in regulation of basal LAMC2 gene expression, a function that is impaired by inflammation.

**Materials and methods**

**Patients and tissue sample**

Forty-nine individuals underwent a routine sigmoidoscopy or colonoscopy as part of their clinical evaluation and were included into the study: patients with quiescent UC (n=10), patients with mild disease activity (n=10), patients with moderate disease activity (n=10), patients with severe disease activity (n=9), and healthy control individuals (n=10) (i.e., patients undergoing an endoscopy due to gastrointestinal symptoms but where all clinical and paraclinical investigations subsequently turned out to be normal). All patients had their diagnosis verified by well-established criteria (Danese and Fiocchi, 2011) and were at the time of enrolment graded in accordance with the Mayo score (Schroeder et al., 1987): a score of 0–2 with endoscopic subscore of 0 (i.e., no macroscopic inflammation) were considered to have quiescent disease, 3–5 mild UC, 6–10 moderate UC, and 11–12 severe UC. The clinical characteristics are summarized in Table 1. Four biopsies of approximately 15 mg each were obtained endoscopically from the sigmoid colon in all patients. Histologic grading or verification of disease activity or quiescent disease was conducted on parallel biopsies taken within an inch of the first biopsy. The biopsies were immediately placed in RNA-Later solution (Life Technologies, Carlsbad, CA, USA), and following 24 h at 4°C the biopsies were stored at −80°C until RNA and protein extraction.

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

**Cell culture and shRNA-mediated knock-down**

The human colonic Caco-2 cell line (American Tissue Type Culture Collection, Rockville, MD, USA) was cultured as monolayers in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and passaged twice weekly as previously described (Coskun et al., 2010).

Two shRNA clones (shCDX2-I and -II) were constructed by inserting two different shRNA sequences targeting CDX2 mRNA into pLKO.1 puro shRNA expression vectors. The shRNA expression vector (pLKO.1 puro) was a kind gift from Dr. Bob Weinberg (Plasmid #8453, Addgene, Cambridge, MA, USA) (Stewart et al., 2003). A lentiviral control
vector containing scrambled non-target shRNA (pLKO.1 scramble) was used as a negative control, and was a kind gift from Dr. David Sabatini (Plasmid #1864, Addgene) (Sarbassov et al., 2005). Next, stable and specific knock-down was performed. Briefly, 24 h before transduction, 5x10^5 Caco-2 cells were seeded in 24-well plates (NUNC Brand). After 24 h, the medium was replaced with sterile filtered viral supernatant with either scramble shRNA or CDX2 shRNA expressing lentivirus in DMEM supplemented with 10% heat-inactivated FCS, and transduced for 24 hours. The supernatant was replaced with fresh DMEM with 10% heat-inactivated FCS and puromycin (15 μg/ml; Life Technologies, Carlsbad, CA, USA) for 72 hours, and CDX2 knock-down was verified by quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

**RNA extraction and qRT-PCR**

Total RNA from Caco-2 cells or human colonic tissue was extracted using the NucleoSpin columns (Macherey-Nagel, Düren, Germany) following the manufacturer’s protocol. 200 ng of each RNA sample was used for cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), and all qRT-PCR reactions were performed on a Stratagene Mx3000P thermocycler (Stratagene, La Jolla, CA, USA) using the Maxima SYBR Green qPCR Master Mix (Fisher Scientific, Pittsburgh, PA, USA) according to manufacturer’s instructions. Target gene expressions were normalized to the expression of human Ribosomal Protein Large P0 (RPLP0) serving as reference gene (Dydensborg et al., 2006).

**Reporter gene assays**

One day prior to transfection, Caco-2 cells were seeded in 24-well plates at a density of 5x10^4 cells/well and transiently transfected the following day as described previously (Coskun et al., 2012). The LAMC2 luciferase reporter constructs were: pGL3-LAMC2 containing the 1.2 kb human LAMC2 promoter region upstream of transcription start site (Olsen et al., 2000), and pGL3-LAMC2+Enhancer containing the 1.2 kb LAMC2 promoter and the enhancer region at chr1:183,149,939–183,150,336 (hg19) (Boyd et al., 2014). The full-length human CDX2 expression plasmid was generated by cloning the PCR amplified wild-type human CDX2 cDNA in the EcoR1 site of pHIV-dTomato (a kind gift from Dr. Byan Welm, Plasmid #21374, Addgene) for expression under regulation of constitutively active EF1a promoter. In all experiments the total quantity of DNA was adjusted to equivalence with pBluescript SK+ plasmid (Stratagene). Forty-eight hours post-transfection, cells were harvested and lysed; luciferase and β-Galactosidase

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activities were determined using the Dual Light system (Perkin Elmer, Waltham, MA, USA) according to manufacturer’s instructions. Reporter gene activities were presented as relative fold induction.

**Chromatin immunoprecipitation assays**
Caco-2 cells to be used for chromatin immunoprecipitation (ChIP) were cultured for 5 days after confluence and stimulated with TNF-α (10 nM, R&D Systems, Minneapolis, MN, USA) or left unstimulated for 24 hours. Cross-linking and sonication were performed as described previously (Boyd et al., 2009). For ChIP analysis on purified colonic cells, colonic tissue had to be prepared before cross-linking. We obtained histologically healthy specimens of fresh mucosal tissue (~1000 mg) from sigmoideum from patients undergoing colonic resection at the Department of Pathology, Herlev Hospital, Denmark. The tissue specimens were snap frozen in liquid nitrogen immediately after resection. Then, the specimens were rinsed with cold PBS and were gently crushed in a mortar containing liquid nitrogen and the pulverized frozen tissue was cross-linked with a final concentration of 1% (v/v) formaldehyde (with 1 µl/ml protease inhibitor) in a small plate for 30 min at room temperature and quenched with 0.125 M glycine for 5 min. Hereafter, the ChIP-protocol was performed as described previously (Coskun et al., 2012). Briefly, immunoprecipitation was done in four replicates and performed overnight at 4°C with an antibody specific for either human CDX2 (α-CDX2 clone CDX2-88; BioGenex Laboratories Inc, Fremont, CA, USA) or as a negative control an antibody specific for the influenza hemagglutinin (HA) epitope (rabbit polyclonal α-HA; Santa Cruz Biotechnology Inc, Heidelberg, Germany). Immunocomplexes were recovered with 50 µl protein A/G beads (Invitrogen). Purified amount of immunoprecipitated DNA was compared to input DNA, corresponding to non-immunoprecipitated sheared cross-linked chromatin through analysis by qPCR. The primers used to amplify the human genomic sequence of LAMC2 promoter were: forward 5’-CAGGTGAGTCACACCCTGAA-3’ and reverse 5’-CGGTTGGTAGGTTCTACTCT-3’ (MWG, Ebersberg, Germany). Quantification of the ChIP-DNA was done using the method described by Frank et al. (Frank et al., 2001).

**Immunohistochemical staining**
Three-micrometer representative sections of formalin fixed and paraffin-embedded biopsies were deparaffinized and pretreated using EnVision FLEX Target Retrieval Solution (DAKO, Glostrup, Denmark). For consistency the tissue sections were processed using the automated DAKO Autostainer LINK according to standard protocols. The

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following antibodies were used: anti-human LAMC2 (1:900, rabbit polyclonal, Sigma–Aldrich, St Louis, MO, USA) and anti-human CDX2 (1:300, mouse monoclonal, DAK-CDX2, DAKO). The slides were counterstained with Mayer’s hematoxylin before viewing. Serial sections were used and immunohistochemical (IHC) staining in the cytoplasm and in the basement membrane was considered positive for LAMC2 whereas nuclear staining was required for CDX2 to be positive.

Statistical analysis
Groups were compared using two-sided Mann-Whitney U-tests and multiple comparisons using Kruskal–Wallis test or Dunn’s multiple comparisons test, and the results were considered significant when $P$-values < 0.05. Spearman’s correlation analysis was used to test for correlations between mRNA and Mayo endoscopic score as well as the overall clinical Mayo score.

Results
The LAMC2 gene activity and endogenous mRNA expression is regulated by CDX2 in Caco-2 cells
To determine whether CDX2 regulates the basal LAMC2 gene activity, a luciferase reporter gene driven by the LAMC2 promoter with and without the upstream LAMC2 enhancer identified previously (Boyd et al., 2014) was transfected into Caco-2 cells without or with concomitant CDX2 expression plasmid. Over-expression of CDX2 increased the luciferase activity driven by the enhancer-less construct of LAMC2 by ~2.5-fold ($P$=0.028) relative to cells transfected with the LAMC2 reporter vector alone, whereas the LAMC2 promoter with enhancer construct gave a 4-fold increase ($P$=0.006) in reporter signal when co-transfected with CDX2 expression plasmid relative to cells transfected with the LAMC2 promoter/enhancer reporter vector alone (Figure 1A).

To further corroborate our hypothesis that CDX2 regulates endogenous LAMC2 expression, we performed shRNA-mediated knock-down of endogenous CDX2 in Caco-2 cells. Two different lentivirus-delivered shRNA constructs against CDX2 were used for transduction and both constructs silenced the CDX2 mRNA expression by more than 35% as compared to control cells (Figure 1B). Consistently, the LAMC2 mRNA levels were
more than 60% lower in CDX2 knock-down cells as compared with scrambled control cells (Figure 1B), confirming that CDX2 is involved in maintaining endogenous LAMC2 expression in Caco-2 cells.

**Interaction of CDX2 with gene regulatory elements in the LAMC2 promoter**

We previously identified a TNF-α-responsive enhancer ~4kb upstream of the main LAMC2 transcription start site (Boyd et al., 2014). This region overlaps a single RELA (p65 subunit of NF-κB) ChIP-seq peak (The ENCODE Consortium., 2012) (Figure 2A). Moreover, genome-wide CDX2 ChIP-seq data extracted from Boyd et al. (Boyd et al., 2010) revealed a CDX2-ChIP peak in the proximal LAMC2 promoter in Caco-2 cells, in which a potential CDX2-binding site strongly was identified (Figure 2A and B). Thus, to further analyze whether LAMC2 is a direct target of CDX2 also in the native chromatin environment, we performed ChIP experiments with Caco-2 and with cells purified from human colonic tissue samples. In ChIP assays on chromatin obtained from Caco-2 cells, qPCR amplification of the anti-CDX2 antibody immunoprecipitated DNA showed a significant enrichment of the LAMC2 promoter (19-fold compared to the negative control, \( P=0.004 \)) (Figure 2C). This shows that the LAMC2 promoter is bound by CDX2 in Caco-2 cells. In purified colonic cells, the LAMC2 promoter was also significantly enriched in anti-CDX2 ChIP assays, albeit with a lower fold change (1.7-fold, \( P=0.037 \)) (Figure 2C).

Because we previously reported that TNF-α reduces the expression of CDX2 and the interaction of CDX2 with its target genes (Coskun et al., 2012; Coskun et al., 2014), we investigated the effect of TNF-α on the physical interaction between CDX2 and the LAMC2 promoter, by performing a ChIP assay on Caco-2 cells stimulated without or with TNF-α. As shown in Figure 2C, stimulation of cells with TNF-α significantly reduced the enrichment of CDX2-bound LAMC2 promoter (0.55-fold, \( P=0.028 \)) compared to untreated cells.

**CDX2 and LAMC2 expression are inversely correlated in inflamed colonic mucosa**

Studies of CDX2 expression in patients with UC have shown strong reduction of CDX2 expression in inflamed mucosa, whereas LAMC2 expression has been reported to be up-regulated in active UC (Coskun et al., 2012; Toedter et al., 2012). Accordingly, we examined the correlation between CDX2 and LAMC2 expression in serial sections from human colonic biopsies of healthy controls, quiescent UC as well as specimens from flaring UC by IHC. CDX2 showed a homogeneous and strong nuclear immunostaining in epithelial cells from healthy individuals and quiescent UC, which was substantially
decreased in active UC (Figure 3). A weak cytoplasmic staining in lamina propria and epithelial cells as well as in the basement membrane in samples from healthy individuals and quiescent UC was observed for LAMC2. However, an increased cellularity with increased immunoreactivity of LAMC2 was observed in active UC (Figure 3). Although the total number of LAMC2-positive cells increased in active UC, we specifically observed an increased LAMC2 reactivity in those particular epithelial cells expressing CDX2, suggesting an inverse correlation between epithelial CDX2 and LAMC2 expression in colonic inflammation.

To confirm this at the molecular level, we determined the mRNA expression levels of CDX2 and LAMC2 in colonic biopsies and investigated their expression patterns in healthy controls and UC samples as a function of disease activity. The epithelial expression of CDX2 mRNA levels were comparable in healthy mucosa and quiescent UC ($P=0.6495$), whereas the level decreased significantly in the mild ($P=0.0076$), in the moderate ($P=0.0037$), and in the severely active ($P=0.0004$) UC groups as compared to healthy controls (Figure 4A). In contrast to CDX2 levels, LAMC2 mRNA levels increased significantly in groups of moderate ($P=0.0057$) and severely active ($P=0.0002$) UC as compared to healthy samples (Figure 4A). Thus, an inverse correlation of CDX2 and LAMC2 mRNA levels were found as the severity of colitis increased (overall $P=0.0002$; Kruskal-Wallis test), supporting the expression pattern determined by IHC staining (Figure 3). Moreover, while CDX2 mRNA correlated negatively to the endoscopic score ($r = -0.49$, $P=0.0004$) and to the grade of disease severity ($r = -0.60$, $P<0.0001$), the LAMC2 mRNA level was positively correlated to the endoscopic score ($r = 0.48$, $P=0.0004$) and to the overall Mayo score ($r = 0.51$, $P=0.0002$) (Figure 4B and C).
Discussion

The intestinal epithelium is critical for the pathogenesis of UC. Laminins are a defining component of epithelial basement membranes and are essential in tissue homeostasis. Dysregulated laminins seem to be involved in the pathogenesis of UC, although current knowledge of laminins contribution to UC is very limited (Spenle et al., 2012). Moreover, aberrantly expression of epithelial-specific genes may affect the intestinal permeability, which leads to penetration of the intestinal mucosa by commensals and results in an overactive immune response, all of which harms the mucosal barrier (Coskun, 2014a; Koch and Nusrat, 2012). In fact, we have previously revealed that the expression of CDX2 becomes significantly down-regulated during inflammation in patients with UC (Coskun et al., 2012). Additionally, other studies have reported an increased expression of LAMC2 in patients with UC (Bjerrum et al., 2014; Toedter et al., 2012). In agreement with these findings, exposure of Caco-2 cells to TNF-α down-regulates the expression of CDX2 and enhances the expression of LAMC2 (Boyd et al., 2014; Coskun et al., 2012). Moreover, TNF-α-mediated modulation of the CDX2 promoter and the LAMC2 enhancer activity is NF-κB-dependent and accompanied by their reduced or enhanced transcript and protein expression, respectively (Boyd et al., 2014; Coskun et al., 2014). Based on these findings, we hypothesized that CDX2 regulates the basal gene expression of LAMC2 in human IECs, however, this regulation may be impaired in inflammatory conditions such as UC.

In the present study, we demonstrated that the basal LAMC2 gene activity is regulated by CDX2 in vitro. We further revealed an inverse effect of inflammation on CDX2 and LAMC2. The data presented here provide a novel insight into how CDX2 is implicated in the transcriptional regulation of LAMC2 in IECs, a function that is impaired in active UC where a high level of TNF-α is present.

Through functional reporter assays we demonstrated that overexpression of CDX2 in Caco-2 cells increased the basal activity of the LAMC2 promoter and increased even stronger the activity of the LAMC2 promoter/enhancer reporter construct. However, transcription factor binding analysis only revealed a single putative CDX2-binding site within the LAMC2 promoter region. This indicates that other potential factors contribute to the inductive effect of CDX2 on the LAMC2 promoter/enhancer reporter construct – possibly through their interaction with the enhancer region. These findings suggest that CDX2 is involved in the regulation of LAMC2 transcriptional activity. Next, we used two CDX2-specific shRNAs to knock-down the endogenous expression of CDX2 and demonstrated that knock-down of CDX2 leads to a significant reduction in the LAMC2 mRNA expression, confirming that CDX2 is involved in maintaining LAMC2 gene
expression in Caco-2 cells. In consistent with other studies (Olsen et al., 2013; Verzi et al., 2010), we were not able to achieve more reduction of CDX2 expression in Caco-2 cells as total CDX2 depletion causes cell death (Verzi et al., 2010). Moreover, the ChIP assays revealed that CDX2 interact with the LAMC2 promoter in vivo, not only in Caco-2 cells, but also in purified human colonic epithelial cells. Together with the results from CDX2 knock-down cells and the luciferase reporter assays, these findings reveal that CDX2 directly binds the LAMC2 promoter and contributes to its expression in the gut at basal conditions. Additionally, we demonstrated that TNF-α treatment suppressed the CDX2 binding to the LAMC2 promoter, and these findings are consistent with our previous observation that TNF-α treatment represses the CDX2 expression and CDX2’s binding to target genes (Coskun et al., 2012; Coskun et al., 2014).

Finally, we revealed an inverse effect of inflammation on the endogenous expression of CDX2 and LAMC2. While CDX2 expression decreased in active UC, the expression of LAMC2 increased markedly. Although, the total number of LAMC2-positive cells increased in active UC, we specifically observed an increased LAMC2 reactivity in those particular epithelial cells expressing CDX2. We demonstrated in UC with mild, moderate, or severe disease activity, that CDX2 expression markedly decreased, while LAMC2 expression increased significantly. Indeed, while LAMC2 mRNA levels correlated positively with endoscopic subscore and the overall Mayo score, CDX2 mRNA was negatively correlated to disease severity. These data further provide evidence that mucosal inflammation has an opposite effect on CDX2 and LAMC2 expression and support the findings in our in vitro experiments. Accordingly, based on the data presented here as well as our previous investigations it is plausible to speculate that pro-inflammatory TNF-α-driven activation of NF-κB impairs the expression and function of CDX2, and takes over the transcriptional regulation of LAMC2 during inflammation through the LAMC2 enhancer (Figure 5).

In summary, we have revealed that CDX2 regulates the basal gene expression of LAMC2 in IECs. Moreover, these data provide novel insight into the molecular regulation of LAMC2 in IECs and in inflammation in response to the pro-inflammatory cytokine TNF-α.

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**Conflict of interest:** No conflicts of interest.
References


Figure Legends

Figure 1. CDX2 regulates LAMC2 transcriptional activity and endogenous LAMC2 expression. (A) Caco-2 cells were transiently transfected with the human LAMC2 promoter reporter construct (pGL3-LAMC2) or LAMC2 promoter and enhancer reporter construct (pGL3-LAMC2+Enhancer) alone (white bars) or together with full-length human CDX2 expression vector (black bars). Values are presented as relative to pGL3-LAMC2 and presented as fold induction. Represented as medians with interquartile ranges (n=4), *P<0.05, **P<0.01. (B) Quantitative RT-PCR analysis showing reduced expression of CDX2 and LAMC2 mRNA in Caco-2 cells transduced with two different lentiviral vectors expressing shRNAs for human CDX2 mRNA (shCDX2-I and -II) relative to the stable reference gene, RPLP0. A lentiviral control vector (−) containing scrambled shRNA was used as a negative control.

Figure 2. The gene regulatory region of LAMC2 and its interaction with CDX2. (A) A UCSC genome browser overview (Rhead et al., 2010) (hg19) of the LAMC2 transcription start site and the recently identified upstream enhancer region containing one single RELA-binding site (p65 subunit of NF-κB) (Andersson et al., 2014; Boyd et al., 2014). Moreover, CDX2 ChIP-seq data from Caco-2 cells, histone marks, vertebrate conservation regions, as well as DNase sensitivity site peaks are shown (Boyd et al., 2010; The ENCODE Consortium., 2012). (B) Position-weight matrix of the CDX2-binding motif [ID: MA0465.1] from the JASPAR database (http://jaspar.genereg.net/) (Mathelier et al., 2014). The predicted CDX2-binding site [chr1:183,156,254-183,156,264 (hg19)] identified in the CDX2 ChIP-seq peak region of the LAMC2 promoter in Caco-2 cells is displayed below the sequence logo. (C) CDX2 ChIP in Caco-2 cells, colonic cells purified from human tissue samples, and Caco-2 cells in the presence or absence of pro-inflammatory TNF-α (10 nM). An anti-CDX2 antibody (white bars) and an anti-HA antibody (black bars), as a control for non-specific immunoprecipitation, were used. Immunoprecipitates were analyzed with primer pairs located within the CDX2 ChIP-seq peaks in the LAMC2 promoter region (shown in (A)), and measured by qPCR. The relative enrichment of CDX2 binding is shown as percentage of total input DNA and is represented as medians with interquartile ranges (n=4). *P<0.05, **P<0.01.

Figure 3. Immunohistochemical staining of CDX2 and LAMC2 in human colonic mucosa. Representative staining of CDX2 (upper panel) and LAMC2 (lower panel) conducted on colonic specimens from healthy controls, quiescent UC, and active UC patient samples.
The expression of CDX2 is limited to the nucleus of colonocytes; whereas LAMC2 is localized to the cytoplasm and to the basement membrane. Insets are higher magnification views of the indicated area and shows the appearance of LAMC2 at the basement membrane (black arrow). Original magnification ×200 or ×400. The evaluation was performed using serial sections from at least three different individuals.

Figure 4. CDX2 and LAMC2 mRNA level in human colonic biopsies and their association to disease severity. (A) CDX2 and LAMC2 mRNA expression patterns measured by qRT-PCR in healthy (n=10) and UC samples graded on a scale of quiescent (n=10), mild (n=10), moderate (n=10), or severe activity (n=9). The mRNA levels were normalized to the level of RPLP0. The data represents values relative to healthy controls presented as boxplots with the median, 25% and 75% quartile values; the bottom and the top whiskers represent the minimum and maximum values, respectively. **P<0.01, ***P<0.001. (B) Correlation of CDX2 and LAMC2 mRNA expression levels to the Mayo endoscopic subscore (0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation, 3 = severe inflammation) and to the (C) overall clinical Mayo score (n=49).

Figure 5. An overview of the proposed role of CDX2 in regulating the LAMC2 gene expression. (A) The basal transcriptional activity of the LAMC2 promoter is positively regulated by CDX2, and during inflammation, TNF-α down-regulates the expression of CDX2 and as a consequence the direct effect of CDX2 on the LAMC2 promoter activity decreases. (B) However, the LAMC2 enhancer region has an additive effect on the basal transcriptional activity of the LAMC2 promoter by yet unknown components. Of note, upon inflammation the activity and expression of LAMC2 is increased significantly indicating that pro-inflammatory transcription factors including NF-κB take over the transcriptional regulation of LAMC2 gene expression through the enhancer region (Boyd et al., 2014).
Figure 1

A

Fold induction

B

Relative mRNA (%)

CDX2 mRNA

LAMC2 mRNA

shCDX2-1

shCDX2-2

shCDX2-1

shCDX2-2
Figure 2
Figure 3
Figure 4
Figure 5
# Table 1

Patient characteristics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls</th>
<th>Quiescent UC</th>
<th>Mild UC</th>
<th>Moderate UC</th>
<th>Severe UC</th>
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<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Gender (Male/Female)</td>
<td>2/8</td>
<td>6/4</td>
<td>3/7</td>
<td>3/7</td>
<td>3/6</td>
</tr>
<tr>
<td>Age, yrs (mean, range)</td>
<td>43 (23-56)</td>
<td>48 (29-68)</td>
<td>36 (27-47)</td>
<td>37 (18-62)</td>
<td>31 (18-59)</td>
</tr>
<tr>
<td>Age at diagnosis (&lt;25/&gt;25 yrs)</td>
<td>–</td>
<td>3/7</td>
<td>3/7</td>
<td>4/6</td>
<td>4/5</td>
</tr>
<tr>
<td>Years with disease (&lt;10/&gt;10 yrs)</td>
<td>–</td>
<td>5/5</td>
<td>7/3</td>
<td>8/2</td>
<td>7/2</td>
</tr>
<tr>
<td>Mayo score (mean, range)</td>
<td>–</td>
<td>0.2 (0-2)</td>
<td>4.2 (3-5)</td>
<td>7.2 (6-10)</td>
<td>12 (11-12)</td>
</tr>
<tr>
<td>Mayo endoscopic score (mean, range)</td>
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<td>0 (0)</td>
<td>1 (0-2)</td>
<td>1.6 (1-2)</td>
<td>2.9 (2-3)</td>
</tr>
<tr>
<td>Smoking/non-smoking</td>
<td>3/7</td>
<td>1/9</td>
<td>0/10</td>
<td>0/10</td>
<td>3/6</td>
</tr>
</tbody>
</table>

UC, ulcerative colitis; yrs, years