The Role of CDX2 in Inflammatory Bowel Disease

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1. Coskun M, Troelsen JT and Nielsen OH. The role of CDX2 in intestinal homeostasis and inflammation. Biochim. Biophys. Acta., 2011;1812:283-289.

2. Coskun M, Olsen AK, Holm TL, Kvist PH, Nielsen OH, Riis LB, Olsen J and Troelsen JT. TNF-α-induced down-regulation of CDX2 suppresses MEP1A expression in colitis. Biochim. Biophys. Acta., 2012;1822:843-851.

3. Olsen AK, Coskun M, Bzorek M, Kristensen MH, Danielsen ET, Jørgensen S, Olsen J, Engel U, Holck S and Troelsen JT. Regulation of APC and AXIN2 expression by intestinal tumor suppressor CDX2 in colon cancer cells. Carcinogenesis, 2013;34:1361-1369.

4. Coskun M, Olsen AK, Bzorek M, Holck S, Engel UH, Nielsen OH and Troelsen JT. Involvement of CDX2 in the crosstalk between TNF-α and Wnt signaling pathway in the colon cancer cell line Caco-2. Carcinogenesis, 2014; In Press.

BACKGROUND

Introduction

The intestinal epithelium is the most vigorously self-renewing tissue of adult mammals and consists of four well-characterised types of differentiated cells: absorptive enterocyte cells (or colonocytes in the large intestine), mucus-secreting goblet cells, enteroendocrine cells and antimicrobial peptide producing Paneth cells (specialised cells in the epithelium of the small intestine) [1,2]. Three additional subtypes of intestinal epithelial cells (IECs) have been discovered recently: M cells, cup cells, and Tuft cells [3]; however, their functions remain largely unknown. The continuous renewal of the intestinal epithelium causes a number of unique challenges. Thus rates of intestinal cell production must be precisely balanced by cell loss. Perturbations in this balance will compromise epithelial barrier function or, alternatively, result in the development of intestinal disorders [4]. Cell proliferation and differentiation are thus tightly controlled in the normal intestinal epithelium. Various genes and transcription factors may take part in this process, in which some are up-regulated and others are down-regulated. One of these well-studied factors is the *Caudal*-related homeobox transcription factor 2 (CDX2). CDX2 is a nuclear transcription factor that is essential for regulating genes related to epithelial functions [5-11] and controlling the balance between differentiation and proliferation of IECs [12]. Thus loss of accurate control of CDX2 expression has been demonstrated to cause a serious disruption in the mucosal architecture, leading to intestinal diseases and developmental disorders. In addition, accumulated knowledge indicates that CDX2 may be pivotal in intestinal inflammation. In fact, a linkage between the key pathways involved in inflammation and regulators of homeostasis is often seen [13,14], supporting the hypothesis that there might be a connection between intestinal inflammation and CDX2 expression.

This PhD thesis explores the role of CDX2 in inflammatory bowel disease (IBD) and investigates the impact of pro-inflammatory pathways on CDX2 expression and its target genes.

CDX genes in intestinal development and homeostasis

The homeobox gene, *Caudal*, was originally identified in *Drosophila* [15], but subsequently other *Caudal* homologue transcription factors that have pivotal roles in intestinal epithelial development and maintenance were identified in a wide array of organisms [5,16-18]. Three *Caudal* homologue genes (*CDX1*, *CDX2*, and *CDX4*) have been identified in mammals. They are expressed during embryonic development, and they contribute to axial patterning [19-22]. In adult mammals the CDX1 and CDX2 homeoproteins have been found to be intestinal transcription factors that regulate homeostasis of the continuously renewing intestinal epithelium. The role of CDX4 in adults is, however, yet unknown. During adulthood, the *CDX1* and *CDX2* genes seem to be differently expressed in the IECs and along the crypt-villus axis [23]. The expression of CDX1 is restricted to the proliferating cells of the crypt compartment [24], whereas CDX2 is found in all epithelial cells located in the crypt-villus epithelium of the small intestine and colon [5,25]. However, CDX2 is active in differentiating enterocytes [26]. By transactivating the promoters of several intestine-specific genes, both CDX1 and CDX2 may be involved in the regulation of proliferation and differentiation of IECs [12,27,28]. In fact, CDX2 is directly involved in the activation of some of the genes characteristic for enterocytic functions such as the *Sucrase-isomaltase*, *Lactase-phlorizin hydrolase, Calbindin-D9K* and *Hephaestin* genes [5,9,10,29].

In the mouse embryo, the first stage of Cdx1 expression is from E (embryonic day) 7.5 to E12, with early expression in the ectoderm and mesoderm of the primitive streak and later in the developing neuroectoderm, somites and developing limb buds [30]. Cdx2 expression begins as early as E3.5, and is confined to the trophectoderm, and persists in the extra-embryonic ectoderm. From E8.5 on, Cdx2 is expressed in the posterior gut endoderm, neural tube and tail bud [31]. By E12.5, the expression of Cdx2 is restricted to the endoderm of the gut [19,32]. The expression of both Cdx1 and Cdx2 increases significantly during the transformation of endoderm into a columnar epithelium (E14–E17) [23].

To directly address the function of CDX2 during early development, transgenic models have been investigated. In loss-of-function assays, mice embryos with inactivated *Cdx2* alleles (*Cdx*$^{-/-}$) experience early lethality due to an implantation failure [33], whereas *Cdx1*-null mice are viable and show anterior homeotic transformation of the axial skeleton [34]. *Cdx2* heterozygotes are viable and fertile; however, the colon and small intestine shows *Cdx2*-deficient lesions with gastric-like epithelium [35]. This has been further supported by conditional homozygous *Cdx2* knockout mice created by Gao *et al.* [36]. These mice had an abnormal colon as the colonocytes differentiate into a gastric rather than an intestinal phenotype, and thus the mice exhibit loss of intestinal morphology. Additionally, gain-of-function models, ectopic expression of *Cdx2* in the stomach of transgenic mice triggers intestinal-like heterodifferentiation of the gastric mucosa, supporting the notion that *Cdx2* is critical in both intestinal cell differentiation and in maintaining the intestinal phenotype [37,38]. Likewise, it has been demonstrated recently that conditional knockout of *Cdx2* in adult small intestinal epithelium or, specifically in stem cells results in an inability of the cells to differentiate into a normal intestinal lineage due to loss of the ability to replace Paneth cells [39] and to produce the definitive intestinal stem cell niche [40]. Instead, the *Cdx2*-negative crypts form subepithelial cystic vesicles that express gastric genes in an intestinal setting [39]. Finally, CDX2 interacts with significantly more genes in differentiated cells than in proliferating cells [11,41]. Thus CDX2 is necessary for normal development and homeostasis of the intestinal phenotype and is a master regulator of intestinal differentiation in both the developing and the adult epithelium [36,39,42-44].

Owing to the essential role of CDX2 in intestinal development and cell phenotype, the transcriptional gene regulation of *CDX2* has been the focus of numerous studies. At the transcriptional level, *CDX2* expression is positively autoregulated by its own expression [11,45,46], as well as by hepatocyte nuclear factor 4 alpha (HNF4α) [47]. However, the expression of CDX2 is not dependent on methylation of its proximal promoter [48]. By using various transgenic genomic fragments of the mouse *Cdx2* locus, Benahmed *et al.* [49] demonstrated that genomic fragments extending to –9 kb of the transcription start site are required to maintain the expression of Cdx2 in the midgut region of the endoderm into adulthood. They demonstrated a 250-bp region around –8.5 kb that revealed interactions with HNF4α, GATA-binding protein 6, T-cell factor 4 (TCF4) and β-catenin that could synergistically activate the expression of *Cdx2*. Moreover, it has been revealed that the expression of CDX2 can be regulated by post-transcriptional mechanisms by small non-coding microRNAs [50-52] and RNA-binding proteins [53], as well as by post-translational mechanisms (described later). Therefore, loss of this tightly controlled regulation of CDX2 expression has several pathological consequences. Indeed, its expression is often reduced in colorectal cancer (CRC) [54-56], and cell differentiation is poor in tumors that lose CDX2 [57]. Furthermore, loss of *Cdx2* promotes tumor progression in genetically [33], and chemically-induced CRC [58]. Together, these findings attribute a tumour suppressor function to CDX2 in the gut. However, several studies have also reported ectopically expressed CDX2 in human intestinal metaplastic lesions where it is involved in transdifferentiation (reviewed in ref. [59]). Hence, CDX2 is a master differentiation factor not only in normal intestinal cells but also in intestinal cells in aberrant locations such as in intestinal metaplasia of the stomach [38], oesophagus [60], gallbladder [61], and liver [62], where it has been described as an oncogene.

In summary, beyond its essential role during intestinal development and homoestasis, CDX2, is involved in tumourigenesis. However, it is questionable whether CDX2 is a tumour suppressor or an oncogene or whether CDX2 has a dual role during cancer progression depending on the tissue and whether it is normally or ectopically expressed.

This subject is further extended in Study I.

Wnt/β-catenin signalling pathway

One of the major signalling pathways involved in the establishment of intestinal homeostasis is the canonical Wnt/β-catenin pathway. Wnt/β-catenin signalling occurs fundamentally in order to maintain the proliferative compartment of the intestinal crypt and renewal of epithelial stem cells [63]. Wnt is a ligand that can activate the β-catenin-dependent pathway (canonical Wnt signalling) or the β-catenin-independent pathway (non-canonical Wnt signalling) [64,65], but the best-characterised pathways is the canonical signalling pathway. Wnt can signal by interaction with different types of receptors, but the best-characterised receptor is the transmembrane receptor Frizzled [66]. In the canonical Wnt signalling pathway, binding of Wnt to its receptor stabilises the cytoplasmic β-catenin which enters the nucleus and associates with TCF family members to activate transcription of Wnt target genes of importance for cell proliferation. However, in the absence of Wnt signalling, free cytoplasmic β-catenin is bound and constitutively phosphorylated by the destruction complex namely, the scaffold protein Axis inhibition protein (AXIN), adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3), and thereby is targeted for proteosomal degradation [63-66].

The gradient of Wnt signalling along the crypt-villus axis in the intestine provides its essential function as a proliferative mediator with highest activity at the bottom of the intestinal crypts and a decreasing activity toward the lumen [67]. This correlates with the accumulating nuclear-localised β-catenin in the proliferating crypt cell. Indeed, when the Wnt/β-catenin signalling is low, epithelial cells lose their proliferative capacity and differentiate.

Because Wnt and CDX2 are essential to intestinal homeostasis and regulate the balance between proliferation and differentiation of epithelial cells, it is vital that they are tightly regulated. In fact, dysregulated Wnt signalling plays a central role in several human disorders, including IBD and CRC [68]. The initiating event of intestinal carcinogenesis is most commonly dysregulation and mutations of components in the Wnt/β-catenin pathway [69]. CRC is one of the most serious and life-threatening long-term complications of chronic intestinal inflammation [70-72], but the risk of CRC in patients with IBD appears to have decreased over time, possibly owing to improved medical therapies [73]. However, key mediators involved in the link between inflammation and development of CRC are still not fully revealed. Therefore, the interplay between Wnt signalling and CDX2 has

been investigated in several studies. Overproduction of Cdx2 in the small intestine modulates nuclear β-catenin levels, resulting in early epithelial maturation and disruption of the Wnt-mediated differentiation of Paneth cells [42]. As a consequence, production of defensins diminishes and may lead to a decrease in mucosal antibacterial activity as seen in mucosal inflammation [74]. Moreover, studies have shown that CDX2 behaves as a tumour suppressor by inhibiting Wnt signalling and the proliferation of colon cancer cells [75,76]. Indeed, it has been revealed recently that β-catenin stabilisation by pro-inflammatory pathways enhances Wnt signalling and induces de-differentiation of epithelial non-stem cells into tumour-initiating cells [77]. Interestingly, approximately one-third of all *cis*-regulatory regions with potential CDX2-binding sites also contain TCF4 motifs [78].

Thus it is likely that CDX2 and TCF4 may commonly interact with the same *cis*-regulatory regions in colonic cells to determine whether a cell should proliferate or differentiate.

Inflammatory bowel disease (IBD)

The two main entities of IBD, i.e., ulcerative colitis (UC) [79] and Crohn's disease (CD) [80], are characterised by a chronic idiopathic inflammation of the intestine. The inflammation in UC is localised exclusively to the colon, and its symptoms are primarily diarrhoea with bleeding, whereas CD is characterised by segmental transmural inflammatory lesions that might occur anywhere in the gastrointestinal tract. The inflammation in CD is more complicated (e.g., fistulas and abscesses) and frequently presents with diarrhoea, abdominal pain and weight loss.

UC affects an estimated 22.000 persons in Denmark [81]. Despite increased knowledge on its pathophysiology and improvements in medical therapy, about 20–30% of UC patients need surgery, i.e., colectomy, because of an inadequate response or a failure to respond to conventional medical therapy (i.e., mesalamine, glucocorticoids and thiopurines) [79]. These numbers from the prebiologic era might, however, change as the introduction of tumour necrosis factor (TNF) inhibitors (TNFi), e.g., infliximab (IFX), adalimumab (ADA) and golimumab (GLM), have revolutionised the treatment and set new standards for mucosal healing and maintenance of clinical remission [82-84]. IFX is a chimeric monoclonal IgG antibody, whereas ADA and GLM are fully human monoclonal IgG antibodies [85,86]. They act by binding to TNF-α and by inhibiting the binding of TNF-α to its receptors [87,88]. Nevertheless, the clinical efficacy of biologics in UC remains unpredictable, as up to 50% of patients do not respond to TNFi (i.e., primary non-responders) [89,90]. Even among patients having an initial response to TNFi, the response may be lost over time (i.e., secondary non-responders) [89-91].

The precise aetiology of IBD is unknown, but it involves a complex interaction among genetic, luminal and environmental factors that trigger an inappropriate mucosal immune response [92-96]. The importance of genetic susceptibility has been established through genome-wide association studies, which have (up to now) identified 163 loci that are significantly associated with IBD, most of which are associated with both CD and UC [97]. Apart from genetics, the importance of environmental risk factors (e.g., smoking, diet, infections and antibiotics) has been explored and seems to be essential in the pathogenesis of IBD [98]. In particular, changes in the composition of the intestinal microbiota are likely the most important environmental factor in IBD [99-102]. Besides the genetic and environmental impacts, the mucosal immune system plays a central pathogenic role in IBD.

In IBD, the balance between pro- and anti-inflammatory mediators is impaired and results in an excessive activation of host immune response towards a diminished diversity of commensal microbiota [103]. This exaggerated immune response (both the innate and adaptive immune systems) is caused primarily by the infiltration of the lamina propria with innate immune cells (i.e., macrophages, neutrophils, dendritic cells and natural-killer T cells) and adaptive immune cells (i.e., B and T cells) [98,104-106]. This infiltration induces the spontaneous release of pro-inflammatory cytokines such as TNF-α, interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β), IL-6, IL-8 and IL-12, all of which might induce an inflammatory cascade resulting in damage to the mucosal barrier [107]. Indeed, the intestinal barrier is crucial for maintenance of intestinal homeostasis [4,13]. Dysregulation within the epithelial layer, such as increased permeability and abnormalities in interactions between IECs and immune cells, plays a key role in the clinical disease course [108]. Thus IBD is a multifactorial disease thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host causing tissue damage.

Tumour necrosis factor-alpha (TNF-α) signalling

Cytokines, which are small peptide proteins produced by immune cells, facilitate communication between cells and have essential functions in cell development and differentiation. A large number of mammalian cytokines, including interleukins and interferons, modulate intracellular signalling by inducing the Janus kinase/signal transducer and activator of transcription pathway (JAK/STAT) [109], as well as the MAPK pathway [110]. Inflammatory cytokine pathways play a central role in the pathogenesis of IBD, and elevated cytokine levels have been found in these patients [111]. Among the best-studied pro-inflammatory cytokines in IBD is TNF-α. The importance of TNF-α as a key pathological factor in IBD has been highlighted by the successful widespread use of various anti-TNF agents to treat patients with IBD [86]. The TNF-α protein exists in two forms: the transmembrane form (tmTNF-α, 26 kDa) and the secreted soluble form (sTNF-α, 17kDa). When synthesised, homotrimeric TNF-α translocates to the cell membrane where TNF-α-converting enzyme (TACE) releases sTNF-α from tmTNF-α by proteolytic cleavage. In analogy to the cytokine, it has been shown that the transmembrane receptors, namely, TNF receptor type 1 (TNFR1) and type 2 (TNFR2), also can be cleaved off the cell surface by TACE to become circulating soluble forms − sTNFR1 and sTNFR2 − where they can act as non-signalling 'neutralising' receptors for TNF-α [112].

TNF-α is secreted by several cell types (e.g., monocytes, macrophages, lymphocytes, neutrophils and epithelial cells) [113]. The biological activity of TNF-α is mediated by its binding to TNFR1 and TNFR2 [114]. TNFR1 is broadly expressed in various cell types, whereas expression of TNFR2 is limited to monocytes and lymphocytes. After binding to the receptor, TNF-α can initiate pro-inflammatory signalling by activating the MAPKs and nuclear factor (NF)-κB pathway. The active MAPK and NF-κB signalling pathways are important for homeostasis, but during inflammation, they induce the up-regulation of several pro-inflammatory factors [110,115].

The MAPK super-family is a member of intracellular serine/threonine-specific kinases that are important in converting extracellular stimuli into a wide range of cellular processes, including cell growth, proliferation, differentiation, migration, inflammation and survival [116-122]. In mammals there are three major constituents of the MAPK superfamily: extracellular signal-regulated kinases (ERKs) (ERK1/2 or p42/p44), c-Jun N-terminal kinases (JNKs) (JNK1/2/3), and the p38 MAPK family [123,124]. The MAPK signalling cascade is activated in response to a diverse

range of stimuli, including growth factors, cytokines and hormones. Activation of specific MAPKs involves phosphorylation and activation of upstream kinases.

Consistent with their critical roles in various key cellular activities, the MAPK signalling pathways have been implicated in the pathogenesis of several human diseases [125-127]. In response to pro-inflammatory cytokines, MAPKs mediates the transcription and activation of various transcription factors that regulate genes involved in IBD [110], and an increased expression of MAPKs has been found in IBD patients [128,129]. Through these transcription factors, MAPKs occupy a pivotal role in the expression and activation of pro-inflammatory cytokines, e.g., IFN-γ, TNF-α, IL-1β, and IL-8, at the transcriptional level and the translational level.

One of the well-studied transcription factors downstream of TNF-α and MAPK signalling is the NF-κB [130,131]. The NF-κB family of transcription factors consists of five mammalian members (p50, p52, p65, cRel, and RelB) [132,133] that can form either homodimers or heterodimers. NF-κB is a key regulator in IBD, as the expression and activation of NF-κB are strongly enhanced in the inflamed gut among patients with IBD [134-138], as well as in experimental colitis models [139]. NF-κB promotes the expression of various pro-inflammatory cytokines including IL-1, IL-2, IL-6, IL-8, IL-12 and TNF-α [115,140]. In addition to enhanced expression and activity of NF-κB in patients with IBD, a constitutive activation of the NF-κB pathway is involved in some forms of malignancies including leukaemia, lymphoma, colon and ovarian cancer [141,142]. NF-κB additionally promotes the expression of a wide variety of genes that are important for the activation of immune responses, including genes encoding chemokines, adhesion molecules, enzymes and genes that facilitate proliferation, tumour promotion and metastatic development [143].

CDX2 in intestinal inflammation

Numerous studies have investigated the importance and role of IECs in intestinal homeostasis and inflammation [144]; however, until now, only limited efforts have been allocated to the role of CDX2 in intestinal inflammation. In one recent study, a diminished CDX2 expression was revealed in UC [145]. On the contrary, another study did not find any correlation between CDX2-positive cells and the degree of inflammation in UC [146]. Thus the source of CDX2 expression in the inflamed mucosa of IBD patients remains to be investigated. However, the link between CDX2 and intestinal inflammation also has been investigated in other species than humans. In *Drosophila*, inhibition of *Caudal* resulted in overexpression of antimicrobial peptide genes which led to increased bacterial growth with elevated apoptosis [147]. Moreover, Calon *et al.* [148] have shown a linkage of CDX2 to inflammation with experimental colitis models and demonstrated that dextran sodium sulphate (DSS) in the drinking water of *Cdx2*$^{+/-}$ mice led to increased intestinal permeability. These animals showed a high susceptibility to the development of DSS-induced acute colitis [148], suggesting that CDX2 is involved in protection against DSS-induced colitis. In fact, several adhesion genes, e.g., *Ll-cadherin*, *E-cadherin* and *Claudin-2 (CLDN2)*, crucial in providing a barrier against bacteria and toxic antigens, have been reported as CDX2 targets [149-151]. Additionally, several studies have reported that *HNF4α*, *Meprin 1A (MEP1A)*, *Peptide transporter 1*, *CLDN2*, and *Mucin 2* are susceptible genes associated with IBD [152-156] and experimental colitis [157-159]. Interestingly, all these genes are CDX2 targets and are important mediators in IECs [11,151,160,161]. Moreover, studies have shown that dysregulation of the differentiation system for correct IEC formation has a crucial role in pathogenesis of UC [14]. Interestingly, CDX2 suppression results in goblet cell depletion in UC [145]. As a consequence, mucin synthesis in active UC is defective, leading to a diminished mucus layer that may increase the invasion of luminal bacteria into the mucosa where they could trigger inflammation [162]. Thus, given the importance of CDX2 in regulating the expression of various genes that govern the proliferation and differentiation of epithelial cells [11,44], and given that CDX2 is necessary for other epithelial-specific transcription factors to work properly [163], it is likely that during chronic colitis, crypt hyperproliferation and remodelling are mediated by dysregulated CDX2 expression.

To date, the regulation of CDX2 in mucosal inflammation is rather unclear. However, a few studies have shown that CDX2 is a downstream target of MAPKs and that its activity is regulated by post-translational phosphorylation [110]. Whereas ERK1/2 reduces the transcriptional activity of *CDX2* [26,164] and the expression of CDX2 [165] by phosphorylating CDX2 at Ser60, phosphorylation of CDX2 by p38 MAPK accompanies cell differentiation and enhances its transcriptional activity [166]. Nonetheless, the p38-mediated phosphorylation of CDX2 depends on the type of stimulus and signalling. This post-translational regulation of CDX2 is in accordance with the essential function of CDX2 in differentiating cells. Indeed, high levels of the phosphorylated and active form of ERK1/2 (p-ERK1/2) is found in proliferating cells, whereas differentiating cells display very low levels of p-ERK1/2. In contrast, the activity of p38 increases in differentiating cells. There is as yet no evidence for a direct regulatory link between JNK and CDX2 activity.

Moreover, one previous study has reported that CDX2 expression is repressed by TNF-α signalling [167]. Their results indicate that TNF-α regulates *CDX2* expression *in vitro* through NF-κB activation in the colon cancer cell line HT29 [167]. The authors have identified two putative NF-κB-binding sites in the *CDX2* promoter, suggesting a direct transcriptional regulation controlled by the balance between p50 and p65 subunits of NF-κB [167]. The authors also have shown that the *CDX2* promoter is able to bind both p50/p50 and p65/p50 dimers, where the activity of the *CDX2* promoter is increased by co-expression with p50, and overexpression of p65 decreased the transcriptional activity of *CDX2* [167]. Surprisingly, the NF-κB p50 subunits do not contain a transactivation domain and therefore cannot activate target-gene expression as a homodimer [168]. However, it has been revealed that p50 can interact with other factors containing a transactivation domain to induce expression of target genes [169,170]. Hence the interaction between NF-κB and CDX2 could be the mechanism linking CDX2 to inflammation by regulating the DNA-binding activity of p50 and p65 in gene expression. Thus it is likely that stimuli from pro-inflammatory cytokines, e.g., TNF-α, activate the NF-κB subunits thereby regulating the CDX2 expression in IBD. In fact, as described previously, inhibition of *Caudal* in *Drosophila* resulted in overexpression of antimicrobial peptide genes, leading to an increased bacterial growth with elevated apoptosis, which is regulated by the balance between *Caudal* and NF-κB [147]. Together these studies suggest that CDX2 is involved in the intestinal inflammatory process and indicate the importance of dissecting the molecular mechanisms underlying the interplay between inflammatory pathways in greater detail, in particular, with respect to the regulation of CDX2 and its relation to inflammation.

This subject is further extended in Study I.

AIMS

The overall objective of this thesis is to explore the role of CDX2 in IBD and to investigate the impact of pro-inflammatory pathways on CDX2 expression and its target genes.

The specific aims are:

1. To investigate CDX2 expression in the inflamed mucosa of patients with IBD and to assess whether CDX2 is regulated by pro-inflammatory cytokines.

2. To reveal whether CDX2 can regulate components of the Wnt/β-catenin signalling pathway.

3. To investigate the influence of TNF-α on β-catenin degradation complex genes and to characterise the molecular mechanism responsible for the TNF-α-mediated down-regulation of CDX2.

MATERIALS AND METHODS

This section provides a brief overview over the methods used in Studies II–IV. (For a more detailed description of the methods, please see Studies II–IV).

Patients and tissue samples (Study II)

Twenty-two individuals underwent a routine colonoscopy because of their clinical condition and were included into the study: patients with active UC (n=6), patients with inactive UC (n=8), and healthy control individuals (n=8). The disease activity of all UC patients was graded in accordance with the Mayo score [171]: a score of ≤2 was graded as disease in remission, and a score >2 (max. 12) was graded as active disease. Six biopsies each of approximately 15 mg were obtained from the descending colon in each patient during the colonoscopy. The endoscopic diagnosis of active or inactive disease was confirmed by histopathology conducted on parallel biopsies taken within an inch of the first biopsy. The biopsies were immediately placed in RNA-Later solution, and following 24 h in the solution at 4°C the biopsies were stored at −80°C until RNA extraction.

Cell culture and treatment (Studies II–IV)

The human intestinal Caco-2 cell line was cultivated as monolayers under standard cell culture conditions at 37°C in an atmosphere of 5% CO_2 and relative humidity of 90% in Dulbecco minimal essential medium supplemented with 10% heat-inactivated faetal calf serum, 4.5 g/L glucose, L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. The medium was changed twice a week and cells were split once a week.

For treatment experiments, $1x10^6$ cells were seeded in 24-well plates and grown to >95% confluence. Cells were then stimulated in medium with or without IFN-γ or TNF-α in the presence or absence of IFX (100 µg/mL) or one of the following chemical inhibitors: tosyl phenylalanyl chloromethyl ketone (TPCK) (NF-κB inhibitor; 100 µM) [172], SC-409 (p38 inhibitor; 10 µM), FR180204 (ERK inhibitor; 30 µM), or dimethyl sulphoxide (DMSO) as a control. In experiments involving treatment with inhibitors, cells were exposed to the inhibitors 1 h prior to addition of TNF-α and subsequently treated with TNF-α (10 nM) for 24 h.

RNA extraction and PCR analysis (Studies II and IV)

Total RNA from isolated colonocytes from tissue samples was isolated as described previously [173], and total RNA from Caco-2 cells was extracted according to the manufacturer's protocol. Then 500 ng of each RNA sample was used for cDNA, and quantitative reverse transcriptase PCR (qRT-PCR) was done. Target-gene expressions were normalised to the housekeeping reference gene human Ribosomal Protein Large P0 (RPLP0), which were amplified in parallel reactions as an internal control.

Protein extraction and immunoblotting (Studies II and IV)

Colonocytes were isolated and lysed with RP1 lysis buffer, and protein extracts were obtained as described in detail earlier [173,174]. Caco-2 cells were lysed in RP1 lysis buffer, and proteins were purified according to the manufacturer's protocol. The primary antibodies were CDX2 (1:1000; mouse monoclonal) and the phospho-p65 subunit of NF-κB (1:1000; rabbit polyclonal). The phosphorylation status of distinct MAPK family members was analysed by using rabbit monoclonal antibodies directed against p38 and ERK1/2 (p44/42). Both MAPK antibodies were used at a dilution of 1:1000. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:20.000; mouse monoclonal) was used as loading control.

Chromatin immunoprecipitation (ChIP) assay (Studies II–IV)

Five days after confluence, Caco-2 cells were either stimulated with TNF-α (10 nM) or left untreated for 24 h. The cells were cross-linked and sonicated as described previously [47]. Chromatin immunoprecipitation (ChIP) was performed as described in detail in Study II.

Briefly, immunoprecipitation was done in four replicates and performed overnight at 4°C with an antibody specific for either human CDX2 (α-CDX2) or an influenza haemagglutinin (HA) epitope (α-HA) used as a negative control. Immunocomplexes were recovered with 50 µL of protein A/G beads. Purified immunoprecipitated DNA and input DNA were analysed by quantitative real-time PCR (qPCR). The primers used to amplify the human genomic sequences of *CDX2* and *MEP1A* were previously described [11], and primers for *APC*, *AXIN2* and *GSK3β* at CDX2 target loci are listed in Study III. Quantification of the ChIP DNA was done using the method described by Frank *et al.* [175].

Immunohistochemistry (Study II)

Four-micrometer sections of formalin-fixed and paraffin-embedded biopsies were deparaffinised and pretreated using EnVision FLEX Target Retrieval Solution (DAKO, Glostrup, Denmark). The tissue sections were processed in an automatic immunohistochemistry (IHC) staining machine using the standard protocols with DAKO Autostainer LINK. The following antibodies were used: cytokeratin 20 (CK20) (1:50, Ks20.8, DAKO M7019) and CDX2 (FLEX CDX2, MxH DAK CDX2). Cytoplasmatic staining was considered positive for CK20, whereas nuclear staining was required for CDX2 to be positive.

Statistical methods (Studies II–IV)

Values are presented as medians (with interquartile ranges). Groups were compared using the Mann-Whitney *U*-test. Two-sided *p*-values of <0.05 were considered significant. Statistical analysis was done using GraphPad Prism, version 5 (GraphPad Software, SD). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Ethics (Study II)

Studies including patient samples (Study II) were approved by the Scientific Ethics Committee of the Capital Region of Denmark.

RESULTS

This section provides a brief overview of the results of this thesis and how the results were interpreted. Results from Studies II–IV that are relevant to the main objective of this thesis are presented. Also, some results are excluded, and some other results that are not published are shown and discussed. The results will be generally discussed and referred to by their figures within this thesis.

Expression of CDX2 in IBD (Study II)

To determine the expression of CDX2 in IBD, mRNA and protein levels in endoscopic biopsies from patients with UC and healthy control individuals were measured using qRT-PCR and Western blot analysis. It was found that mRNA and protein levels of CDX2 correlated significantly with disease activity (Fig. 1). To eliminate the possibility that any changes in mRNA expression in inflamed mucosa are the result of a reduced number of colonocytes in the biopsies, the mRNA expressions where determined as ratios to CK20 mRNA (an established IEC marker). Relative mRNA levels of CK20 were found to remain unchanged in colonic mucosa from healthy individual samples and samples from patients with quiescent and active UC (data not shown), indicating a relatively unaffected number of colonocytes in the three groups. Patients with inactive UC had CDX2 mRNA levels similar to those found in healthy individuals (Fig. 1A). In contrast, expression of CDX2 mRNA was significantly decreased ($p < 0.01$) in the colonocytes of patients with active UC. Consistently, CDX2 protein abundance was significantly decreased in patients with active UC, and quantification by densitometry scanning showed an approximate 40% reduction in CDX2 protein level in patients with active UC (Fig. 1B).

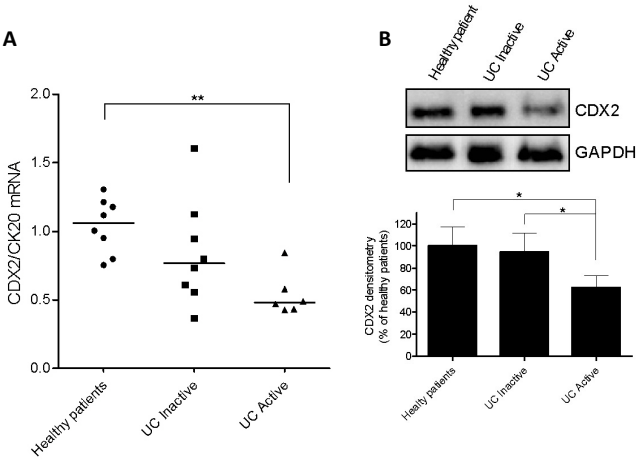

Figure 1. Expression of CDX2 in UC. CDX2 mRNA (A) and protein levels (B) in colonocytes from healthy control individuals and patients with inactive and active UC were measured by qRT-PCR and Western blot analysis. mRNA levels were normalised to RPLP0 and the ratio to CK20 is shown (Study II).

Consistent with the qRT-PCR and immunoblotting analysis of CDX2 expression, IHC staining of CDX2 and CK20 displayed a positive expression in all IECs in samples from healthy individuals and in sample from patients with quiescent UC (Fig. 2A). In patients with active UC, CDX2 expression, however, was markedly decreased, whereas CK20-positive cells were unchanged (Fig. 2A), indicating that the decreased protein levels of CDX2 were caused by the disease activity. Also investigated was the expression of CDX2 in patients with active and quiescent colon CD by IHC analysis, and no difference was found at the cellular level between quiescent and active disease (Fig. 2B).

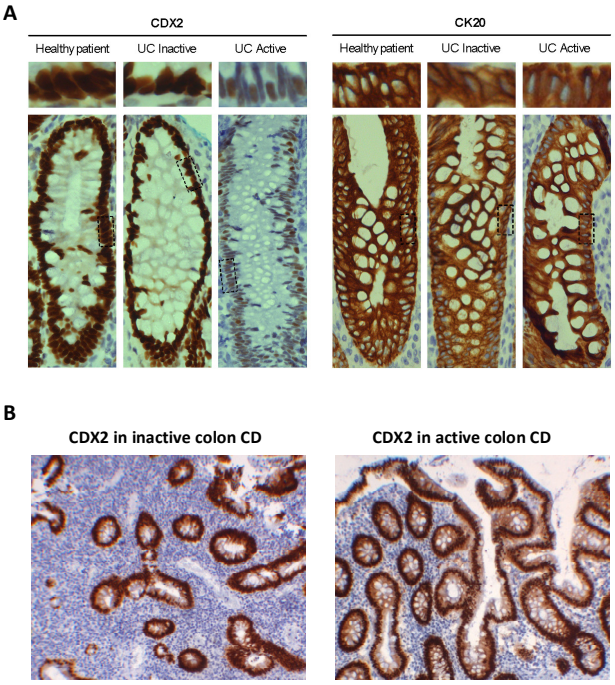

Figure 2. Immunohistochemical staining of CDX2 in IBD. (A) CDX2 protein expression in a healthy individual and in patients with inactive and active UC. CK20 staining is a marker of epithelial cells (Study II). (B) CDX2 expression in patients with inactive and active colon CD.

Expression of MEP1A and TNF-α in colitis (Study II)

It has been demonstrated previously that *MEP1A,* a metalloprotease that is expressed by IECs and hydrolyses a variety of peptide and protein substrates [176-178], is a direct target gene of CDX2 [11]. Indeed, knockdown of *CDX2* significantly suppressed the expression of *MEP1A* transcript by more than 40% and reduced the *MEP1A* promoter activity by approximately fivefold in Caco-2 cells [11]. Consistently, it was confirmed that the *MEP1A* gene promoter is CDX2-responsive in Caco-2 cells (see Study II). Because a recent study has revealed a reduced expression of MEP1A in patients with UC [153], and because the cell-line experiments indicate that *MEP1A* is positively regulated by CDX2, the expression status of MEP1A in patient samples was assessed to evaluate whether MEP1A and CDX2 mRNA levels are correlated. As expected, the *MEP1A* gene transcript was (like *CDX2* levels) significantly reduced in patients with active UC *versus* healthy control individuals ($p < 0.01$), and MEP1A mRNA showed a significant difference between patients with quiescent and active UC ($p < 0.001$; Fig. 3A). The inflamed colon, however, exhibited a significantly increased TNF-α mRNA expression as compared with samples from healthy individuals and quiescent UC patients ($p < 0.001$, respectively; Fig. 3B). These observations indicate a close relationship between CDX2 and MEP1A expression during colitis and interestingly, the expression pattern is inversely correlated with the expression pattern of TNF-α. This also was the case in experimental DSS-induced murine colitis (see Study II).

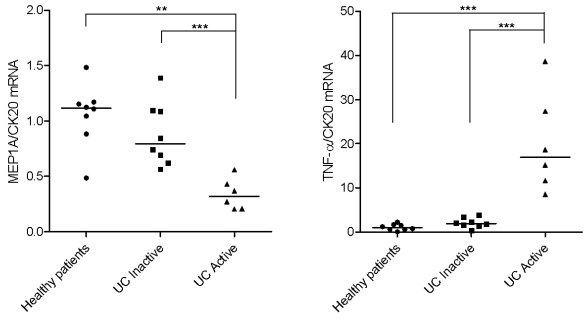

Figure 3. Expression of MEP1A and TNF-α mRNA levels in UC. Expression levels of MEP1A (A) and TNF-α mRNA levels (B) in colonocytes from healthy control individuals and patients with inactive and active UC were measured by qRT-PCR and normalised to RPLP0. Values are shown as ratios to CK20 (Study II).

TNF-α decreases CDX2 and MEP1A expression (Studies II and IV)

To understand the role of CDX2 in epithelial inflammation, the influence of pro-inflammatory cytokines on endogenous CDX2 expression was investigated. Therefore, Caco-2 cells were treated with increasing concentrations of TNF-α (0.1, 1 or 10 nM) for 24 h to induce a TNF-α response. Treatment with TNF-α significantly reduced the expression of CDX2 mRNA and protein levels in a dose-dependent manner with maximal reduction observed at 10 nM (Fig. 4A and B).

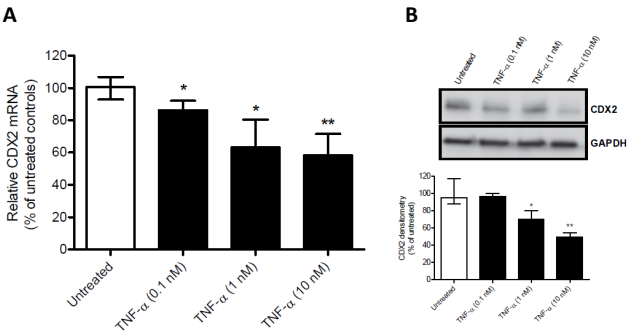

Figure 4. The *in vitro* effect of TNF-α on CDX2 expression in Caco-2 cells. (A) Dose-dependent decrease in CDX2 mRNA measured by qRT-PCR and (B) CDX2 protein expression in TNF-α-treated Caco-2 cells measured by Western blot and densitometric analysis (Study IV).

Having identified that TNF-α reduces CDX2 mRNA in Caco-2 cells, it was subsequently investigated whether TNF-α affects the expression of MEP1A and whether blocking of TNF-α with anti-TNF-α antibody (IFX) could reverse this effect. This antibody was chosen, because it is an effective inhibitor of TNF-α *in vitro* and because it was the first introduced (and therefore the most evidence is available) TNFi for treatment of various inflammatory diseases. As shown in Figure 5, exposure to TNF-α significantly decreased MEP1A expression ($p < 0.001$; Fig. 5B); however, co-treatment of cells with TNF-α and IFX significantly increased both CDX2 and MEP1A expression ($p < 0.01$ and $p < 0.001$, respectively; Fig. 5A and B) compared with cells stimulated with TNF-α alone. Conversely, TNF-α blockade by IFX significantly decreased IL-8 ($p < 0.001$; Fig. 5C), indicating that IFX effectively neutralised the signalling induced by TNF-α in these experimental conditions. Interestingly, exposure with IFN-γ, another pro-inflammatory cytokine, had no effect on CDX2 and MEP1A transcripts. IL-8 is a pro-inflammatory component produced in response to injury and is a well-known target of IFN-γ and TNF-α. In Fig. 5C, mRNA measurements in the medium of IFN-γ- or TNF-α-exposed cells showed a significant increase in IL-8 mRNA expression, indicating an inflammatory state in the cells.

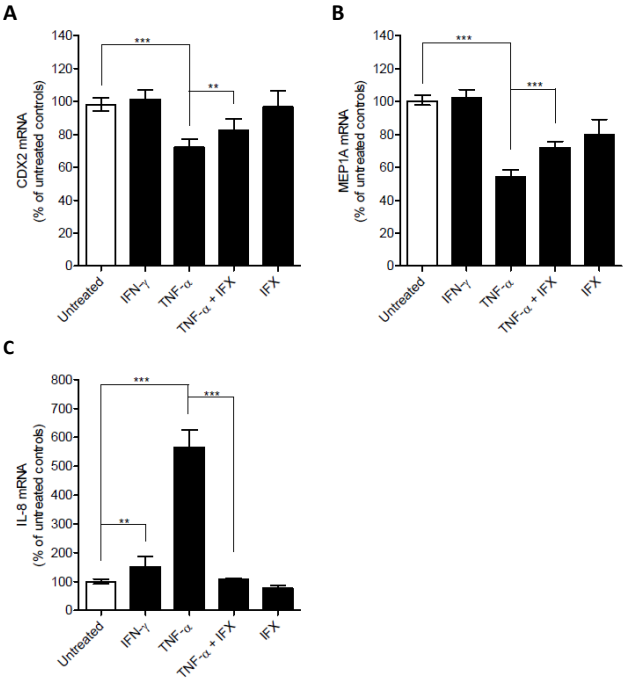

Figure 5. Effect of pro-inflammatory cytokines and/or infliximab (IFX) on endogenous mRNA expression in Caco-2 cells; (A) CDX2, (B) MEP1A, and (C) IL-8 mRNA (positive control) in treated and untreated Caco-2 cells. Cells were treated for 24 h, and mRNA levels were measured by qRT-PCR and normalised to the level of RPLP0 (Study II).

Signalling pathways in the TNF-α-induced suppression of CDX2 (Study IV)

Activation of NF-κB, p38 and ERK1/2 pathways has been demonstrated previously to regulate the expression of CDX2 [26,164-167]. Therefore, the role of these signalling pathways in TNF-α-mediated down-regulation of endogenous CDX2 expression in Caco-2 cells was investigated. The NF-κB inhibitor TPCK effectively reversed the effect of TNF-α on CDX2 mRNA, indicating that a TNF-α-mediated down-regulation of CDX2 mRNA requires NF-κB activation (Fig. 6A). Western blot analysis revealed that TPCK partially reversed the TNF-α-mediated reduction of CDX2 protein expression (Fig. 6B). Inhibition of p38 or ERK1/2 with SC-409 or FR180204, respectively, significantly increased the level of CDX2 mRNA ($p < 0.01$ and $p < 0.001$, respectively) compared with the DMSO control but did have only a minor effect on the inhibitory effect of TNF-α on CDX2 mRNA expression (Fig. 6A). Surprisingly, treatment of Caco-2 cells with SC-409 decreased the CDX2 protein levels but effectively removed the inhibitory effect of TNF-α on CDX2 protein expression (Fig. 6B). Treatment with FR180204 increased CDX2 protein expression but had no effect on the TNF-α-mediated inhibition of CDX2 protein expression (Fig. 6B). Furthermore, the results demonstrated that the inhibitors were effective in preventing the phosphorylation of p38 and ERK1/2, as well as the activation of the p65 subunit of NF-κB by each specific inhibitor (Fig. 6B). However, treatment with a JNK inhibitor (SP600125; 100 nM) had no effect on mRNA and protein levels (data now shown).

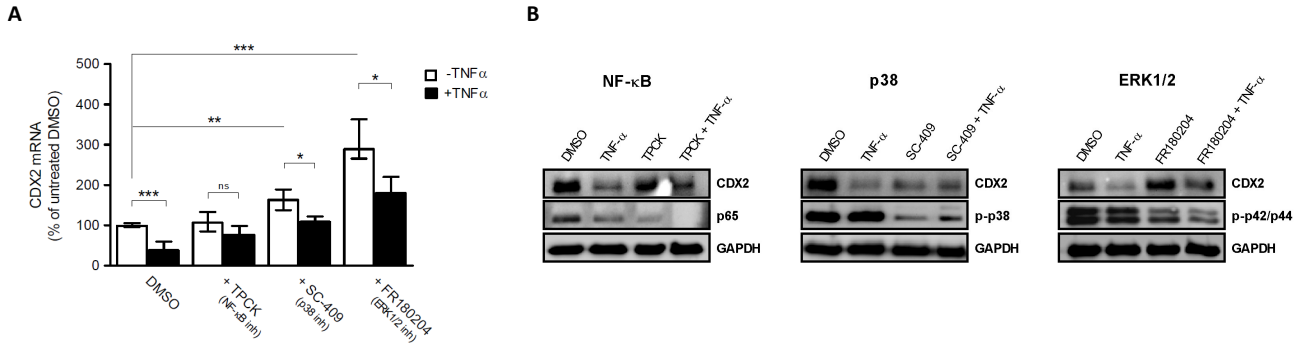

Figure 6. Signalling pathways of TNF-α-mediated down-regulation of CDX2 expression in Caco-2 cells. (A) Endogenous CDX2 mRNA expression (normalised to RPLP0) in untreated cells (*white columns*) and in TNF-α-treated cells (*black columns*) in the absence or presence of signalling inhibitors (TPCK, a NF-κB inhibitor; SC-409, a p38 inhibitor; and FR180204, an ERK inhibitor). (B) Whole-cell extracts from Caco-2 cell monolayers immunoblotted for CDX2, active NF-κB (p65), phospho-p38 (p-p38), phospho-ERK1/2 (p-p42/p44) and GAPDH (Study IV).

CDX2 regulates Wnt signalling components (Study III)

Examining data from a recently published genome-wide CDX2 ChIP-Seq analysis revealed CDX2-binding regions at the upstream putative enhancer elements of *APC* and *AXIN2* genes and at the promoter region of *GSK3β* [11]. To verify these findings, CDX2 ChIP assays were performed in which chromatin–protein complexes were immunoprecipitated with a CDX2-specific antibody or a HA antibody (negative control) in Caco-2 cells. The amounts of co-immunoprecipitated *APC* and *AXIN2* upstream enhancer elements and *GSK3β* promoter DNA were measured by qPCR. The *in vivo* binding of CDX2 to all examined regulatory DNA regions was significantly elevated compared with negative controls (Fig. 7). Furthermore, these *in vivo* findings have been supported by CDX2 overexpression and siRNA-based knockdown of *CDX2* in Caco-2 cells, confirming that *APC* and *AXIN2* are positively regulated by CDX2, whereas regulation of *GSK3β* is more complex (see Study III).

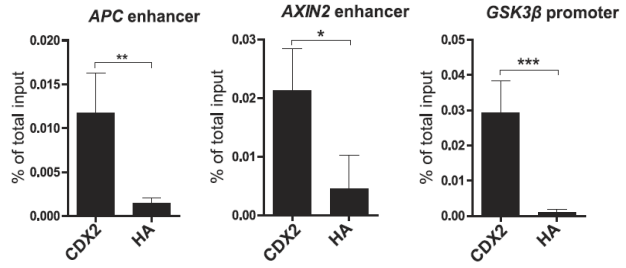

Figure 7. CDX2 binds to gene regulatory regions of *APC* enhancer, *AXIN2* enhancer and *GSK3β* promoter analysed by qPCR using gene-specific primers. Values are shown as a percentage of total input DNA (Study III).

TNF-α down-regulates β-catenin inhibitors (Study IV)

We next investigated whether TNF-α treatment affects the endogenous mRNA level of APC, AXIN2 and GSK3β. A significant reduction of APC, AXIN2, and GSK3β mRNA levels ($p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively) was observed when Caco-2 cells were treated with TNF-α (Fig. 8). Interestingly, the mRNA reduction levels of these targets were almost comparable with the approximately 50% reduction of CDX2 mRNA in TNF-α-treated (10 nM) Caco-2 cells (see Fig. 4).

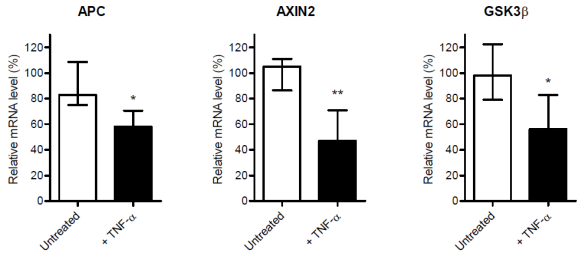

Figure 8. Endogenous mRNA expression levels of APC, AXIN2 and GSK3β in Caco-2 cells treated with or without TNF-α (10 nM for 24 h). Expression levels were measured by qRT-PCR and normalised to RPLP0 (Study IV).

TNF-α suppresses *in vivo* binding of CDX2 to target genes (Studies II and IV)

Based on findings that CDX2 is down-regulated in epithelial cells in inflammation, it was hypothesised that TNF-α-induced down-regulation of CDX2 expression has a functional consequence on the interaction of CDX2 with its target genes. To address this question, the effect of TNF-α on the physical interaction between CDX2 and the gene regulatory regions on target genes was investigated by ChIP analysis. It was demonstrated previously that CDX2 can regulate its own as well as the *MEP1A* expression [11], and luciferase reporter assays have been performed and have demonstrated that TNF-α significantly reduces the transcriptional activity of *CDX2* and *MEP1A* reporter constructs (see Study II), demonstrating a regulation of *CDX2* and *MEP1A* expression at the transcriptional level. Supporting these findings, chromatin-protein complexes from untreated and TNF-α-treated Caco-2 cells were immunoprecipitated with a CDX2 antibody or a HA antibody (negative control). The amount of co-immunoprecipitated CDX2–DNA complexes was measured by qPCR. The *in vivo* binding of CDX2 to the 5' and 3' regulatory elements of the *CDX2* gene decreased significantly ($p < 0.05$) in cells treated with TNF-α (Fig. 9). Furthermore, the binding of CDX2 protein to the *MEP1A* promoter region was significantly suppressed ($p < 0.05$), as shown by reduced co-immunoprecipitated *MEP1A*-specific DNA in TNF-α-treated cells as compared with untreated cells (Fig. 9). Similarly, the enrichment of CDX2-DNA complexes was decreased significantly within *APC* ($p < 0.05$) and *AXIN2* ($p < 0.01$) upstream enhancer elements, and the binding of CDX2 to the *GSK3β* promoter region was reduced significantly ($p < 0.05$) in TNF-α-treated Caco-2 cells as compared with untreated cells (Fig. 9).

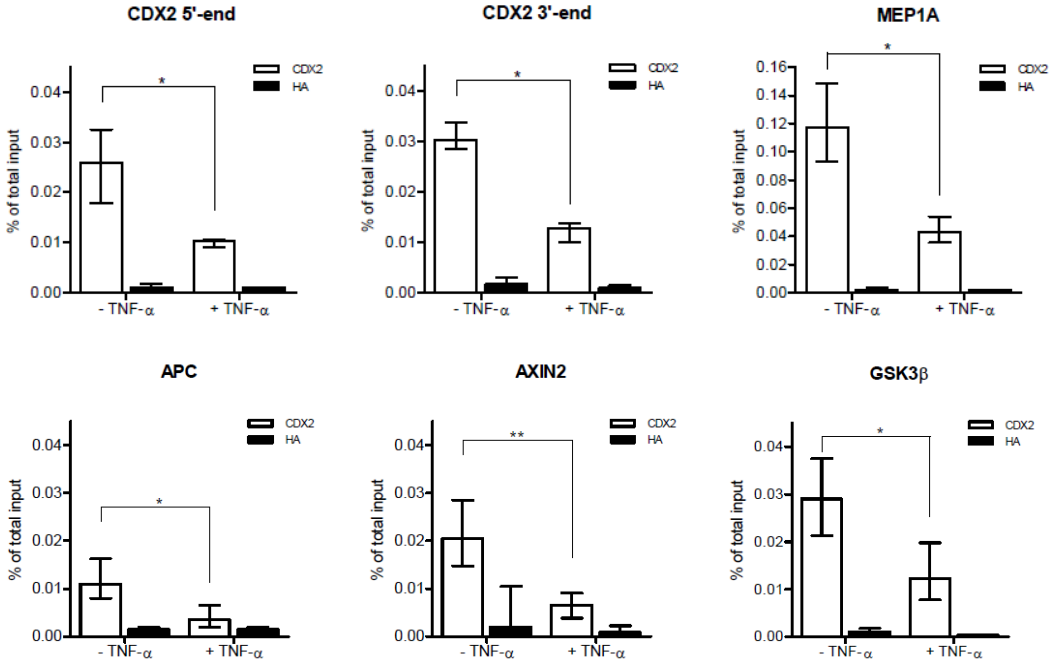

Figure 9. Effect of TNF-α on the *in vivo* binding of CDX2 to gene regulatory regions of CDX2-targets. ChIP assay using a CDX2 antibody (*white bar*) and negative control HA antibody (*black bar*) in Caco-2 cells in the presence or absence of TNF-α. Enrichment was analysed by qPCR using gene-specific primers, and the values are shown as a percentage of total input DNA (Studies II and IV).

TNF-α enhances the expression levels of Wnt target genes (Study IV)

Since, CDX2 regulates components of the β-catenin degradation complex and the regulatory function of CDX2 is suppressed by pro-inflammatory TNF-α, we examined the influence of TNF-α on the expression of Wnt-related target genes via qRT-PCR in Caco-2 cells. Exposure of Caco-2 cells with TNF-α increased the expression of well-known Wnt targets (Fig. 10). The expression of SOX9 ($p < 0.05$), cyclin D1 ($p < 0.05$), cMYC ($p < 0.05$), and HEF1 ($p < 0.01$) mRNA levels were significantly enhanced in Caco-2 cells exposed to TNF-α for 24 h (Fig. 10). These findings demonstrate that Wnt signalling target genes are activated by the pro-inflammatory cytokine TNF-α.

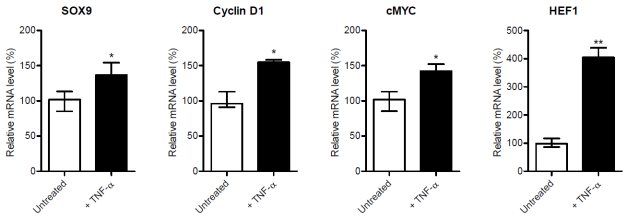

Figure 10. Effect of TNF-α on endogenous expression of Wnt-related target genes in Caco-2 cells. Endogenous mRNA expression levels of SOX9, cyclin D1, cMYC, and HEF1 in cells treated with or without TNF-α (10 nM) (Study IV).

GENERAL SUMMARY AND DISCUSSION

High levels of pro-inflammatory cytokines are present in the gut of IBD patients. These cytokines play a critical role in the pathogenesis of chronic intestinal inflammation, causing dysfunction of the mucosal barrier and intestinal homeostasis. The nuclear homeobox transcription factor CDX2 is of importance for intestinal differentiation and homeostasis and may be a key factor for IBD pathogenesis. Thus, the primary objective of this investigation was to explore the role of CDX2 in the colonic epithelium of patients with IBD and to investigate the impact of pro-inflammatory pathways on the expression of CDX2 and its target genes.

In Study I of this thesis, the importance of CDX2 in intestinal homeostasis and its potential role in inflammation were reviewed. There is clear evidence for implicating the CDX2 factor in modulating a diverse set of cellular processes, including cell proliferation and differentiation, and cell adhesion and migration. On the other hand, what is less clear is the role of this factor in relation to gut inflammation. This study provided the key role of CDX2 in IEC function and contribution to the pathogenesis of IBD and also pointed out which pro-inflammatory pathways might directly or indirectly affect CDX2 expression. Thus inflammatory mediators have been mechanistically linked with the regulation of CDX2 expression, and an altered CDX2 expression can disrupt the mucosa in protecting the host against its luminal components, which might result in an increased expression of inflammatory mediators, potentially leading to episodes of colonic inflammation, as observed in IBD. The subsequent studies, Study II–IV, investigated the expression of CDX2 in colitis and examined the influence of pro-inflammatory cytokines on the expression of CDX2 and its target genes.

To investigate the role of CDX2 in the pathogenesis of intestinal inflammation, we measured the level of CDX2 in IBD. The results show a significantly decreased level of CDX2 in IECs in biopsies from patients with active UC (Study II), whereas CDX2 expression was unchanged in patients with CD. This finding is of clinical importance for several reasons. It indicates that CDX2 is specifically involved in the dysregulation of IEC differentiation in UC and suggest that CDX2 can be used as a mucosal marker to differentiate between active and inactive disease as well as between UC and CD. Hence, further studies should aim at exploiting this paradigm in larger patient cohorts.

Previous investigations have shown a strong reduction in MEP1A expression in patients with UC, and several polymor-

phisms in the *MEP1A* gene have been associated with UC [153]. This gene encodes the α subunit of meprins found as a secreted form or as a membrane-bound form at the brush-border membrane in association with the transmembrane β subunit in IECs where their main function is to cleave diverse substrates such as laminins, tight-junction proteins and cytokines [176-181]. Complementing previous findings by Boyd *et al.* [11], these findings have demonstrated that *MEP1A* is positively regulated by CDX2 and depends on CDX2 activity (Study II). Further, this study confirmed a reduced expression of MEP1A in UC, and the observations supported a close relationship between CDX2 and MEP1A expression during colitis (also in experimental murine colitis). Interestingly the expression pattern was inversely related to the expression pattern of TNF-α.

Intestinal inflammation is a complex process involving the action of a number of cytokines. During active stages of IBD, the levels of pro-inflammatory cytokines such as TNF-α and IFN-γ are up-regulated in the mucosa [98]. Given their protective role in the intestinal epithelium of mice [148,153,158], the aim was to assess whether CDX2 and MEP1A are regulated by pro-inflammatory cytokines. It was revealed that Caco-2 cells in response to TNF-α had significantly decreased endogenous CDX2 and MEP1A expression but not in response to IFN-γ (Study II). In fact, this is not surprising because TNF-α and IFN-γ activate different pathways; TNF-α activates the NF-κB and MAPK pathways, whereas IFN-γ signalling is induced by activation of the JAK/STAT signalling pathways [109,114]. Therefore, the results indicate that down-regulation of CDX2 is not a general consequence of inflammatory signalling in Caco-2 cells and suggest a major role for TNF-α in the suppression of CDX2 and MEP1A expression during inflammation.

As biologic agents with anti-TNF-α antibodies have become an important treatment option in the treatment of IBD during the last decade [86], the effect of IFX treatment on TNF-α-mediated down-regulation of CDX2 and MEP1A was investigated. The *in vitro* data showed that TNF-α neutralisation with IFX induce a significantly higher expression of CDX2 and MEP1A by IECs (Study II). Arijs *et al.* previously examined the gene expression profile of IBD patients before and after IFX treatment [182,183]. Thus, when the *CDX2* and *MEP1A* expression profiles were extracted from the microarray data, both *CDX2* and *MEP1A* gene expressions were significantly up-regulated ($p < 0.01$, respectively) in IFX-treated UC patients compared with their baseline samples. Taken together, the *in vitro* observations of this study support the clinical findings because treatment-naïve UC patients responding to IFX have significant higher *CDX2* and *MEP1A* colonic gene expression levels after therapy. In addition, gene expression profiles from treatment-naïve patients with colon CD have CDX2 expression levels similar to those of controls, further supporting our clinical findings in patients with active colon CD.

The pro-inflammatory cytokine TNF-α signals through multiple intracellular pathways. One of the major signalling pathways used by TNF-α is that of NF-κB [184]; however, NF-κB must be further modified by phosphorylation of its subunits for full activation. Several kinases, including MAPKs, have been implicated in this secondary modification of NF-κB [110]. A previous study demonstrated that pro-inflammatory TNF-α can regulate *CDX2* expression through NF-κB activity in HT29 cells [167]. Moreover, CDX2 is regulated by MAPKs, which are highly active during inflammation [26,164-166]. Indeed, we characterised the molecular mechanism responsible for the TNF-α-mediated down-regulation of CDX2 and demonstrated that the suppressive effect of TNF-α on the endogenous CDX2 expression levels is mediated by NF-κB and partially by p38 MAPK (Study IV). In *Drosophila,* the expression of antimicrobial peptides is regulated by a balance between Caudal (homologues to human CDX2) and NF-κB [147]. Inhibition of *Caudal* increases the expression of NF-κB-induced antimicrobial peptides, which in turn alter the commensally bacteria population and cause apoptosis of IECs. Moreover, reintroducing the expression of *Caudal* restores a healthy microbiota and results in normal host survival [147]. Thus, with this study (Study IV) we provided new insight into the molecular explanation for how TNF-α down-regulates the expression of CDX2 *in vitro*.

Concerning ERK1/2 inhibition (Study IV), our results are in line with the previous findings by Lemieux *et al.* [164] and Krueger *et al.* [165], because inhibition of ERK1/2 increases the endogenous levels of CDX2 mRNA and protein levels. Together these findings support the notion that CDX2 and ERK1/2 are inversely active further underlying the essential role of CDX2 in differentiating cells and the increased ERK1/2 activity sustained in proliferating cells. As mentioned earlier, one of the major signalling pathways involved in maintaining the proliferative compartment of the intestinal crypt is the canonical Wnt/β-catenin pathway [63]. Therefore, we investigated the interaction between CDX2 and Wnt signalling components and demonstrated that CDX2 regulates expression of β-catenin degradation complex genes (Study III). Accordingly, it is likely that CDX2 could regulate the activity of β-catenin degradation complex genes to control the activity of the Wnt/β-catenin signalling pathway and thereby direct the IECs as to whether they should differentiate or proliferate. In fact, this hypothesis is supported by previous investigations showing that CDX2 can inhibit the Wnt signalling and the proliferation of colon cancer cells [75,76].

Following the findings of decreased CDX2 expression in colitis (Study II) and its regulatory function on Wnt/β-catenin signalling components (Study III), we examined the influence of TNF-α on β-catenin degradation complex genes (Study IV). In this study, we found a decreased expression of *APC, AXIN2* and *GSK3β* in Caco-2 cells exposed to TNF-α. These data fit with the TNF-α-induced activation of Wnt/β-catenin signalling in macrophages in gastric cancer cells *in vitro* [185]. Indeed, a recent study by Schwitalla *et al.* [77] also has revealed that TNF-α signalling stabilises β-catenin, enhances Wnt signalling and induces de-differentiation of epithelial non-stem cells into tumour-initiating cells. In fact, those authors have demonstrated that stabilisation of Wnt signalling is associated with the binding of p65/RelA (subunits of NF-κB) to β-catenin [77]. Moreover, it has been previously demonstrated that p38 MAPK can induce β-catenin-mediated signalling by inactivating GSK3β [186]. Hence, based on previous studies and our observations, TNF-α-induced activation of NF-κB and p38 MAPK might enhance Wnt signalling by repressing CDX2 and β-catenin degradation complex genes (Fig. 11). In fact, this will support the clinical findings, as β-catenin recently has been shown to be up-regulated in patients with UC but not CD [187], correlating with the reduced expression of CDX2 in patients with UC (Study II).

In addition to our *in vitro* findings, we confirmed by ChIP assays that TNF-α reduced the binding of CDX2 to gene regulatory regions of target genes. In accordance with previous findings [11], we have demonstrated that CDX2 is able to bind to its own promoter and enhancer region, as well as to the *MEP1A* promoter region (Study II). In this study we revealed that CDX2-binding is highly reduced by the presence of TNF-α. Indeed, this was also the case for the interaction between CDX2 and the gene regulatory regions of *APC*, *AXIN2* and *GSK3β* (Study IV). Thus, the data are in agreement with a previous study suggesting that TNF-α represses CDX2 expression [167], and the present study, to our

knowledge, is the first to report that TNF-α reduces the binding of CDX2 to its target genes.

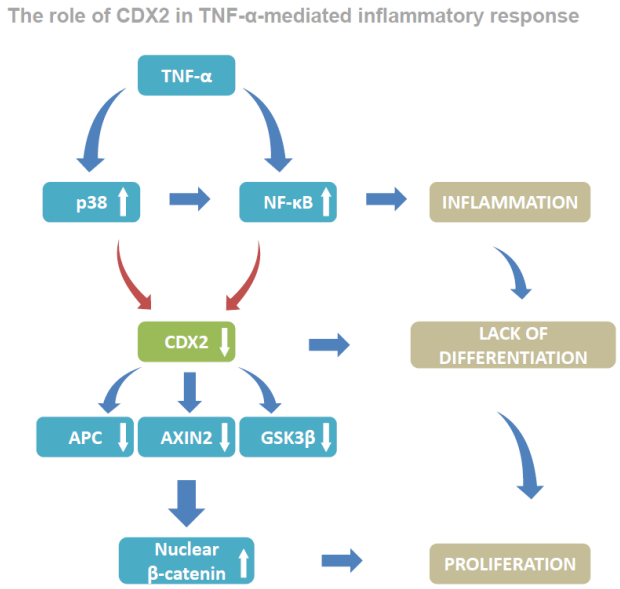

Figure 11. Flow chart summarising the role of CDX2 in the context of our findings in this thesis. Inflammatory response promotes a TNF-α-mediated activation of p38 MAPK and NF-κB pathway that induce intestinal inflammation. The TNF-α-induced response also down-regulates the expression of the transcription factor CDX2 to decrease the transcriptional activity of APC, AXIN2, and GSK3β (essential components of the β-catenin degradation complex), and consequently nuclear β-catenin might accumulate and direct the intestinal epithelial cells to proliferation.

Following the findings of the suppressive effect of TNF-α on CDX2 and on the components of the β-catenin degradation complex, it could be assumed that TNF-α has an effect on the activity of the Wnt signalling. Accordingly, we found an increased mRNA expression level of *SOX9*, *cyclin D1, cMYC,* and *HEF1* – reported as being direct Wnt pathway target genes [188-191] – in Caco-2 cells exposed to TNF-α.

Taken together, it is plausible to speculate that inflammatory cell infiltrates increase extracellular stimuli by TNF-α to induce epithelial proliferation by suppressing epithelial CDX2 expression and increasing Wnt signalling (Fig. 11), thereby causing initiation and development of intestinal tumourigenesis [77].

CONCLUSIONS AND FUTURE DIRECTIONS

The general aim of this thesis was to explore the role of CDX2 in IBD and to investigate the impact of pro-inflammatory pathways on the expression of CDX2 and its target genes. Through this work, we have found a decreased CDX2 expression specifically in UC, and we have shed light on some of the molecular pathways of importance involved in the inhibitory effects of TNF-α. We have provided novel insight into the molecular regulation of genes involved in the β-catenin degradation complex by CDX2 and in response to the pro-inflammatory cytokine TNF-α. In light of our findings, we propose that a dysregulated CDX2 expression is involved in the pathogenesis of UC, and that CDX2 is involved in the crosstalk between TNF-α and the Wnt signalling pathway. This might be of importance for understanding the link between intestinal inflammation and tumourigenesis.

Investigation of the expression of CDX2 in patients with IBD allowed us to find CDX2 specifically decreased in colonocytes from patients with UC. Therefore, it would be obvious to investigate the expression of CDX2 in larger patient cohorts to reveal if CDX2 expression can be used to differentiate between active and quiescent UC as well as between UC and CD. Our *in vitro* experiments revealed that the expression of CDX2 and its ability to interact with target genes were repressed by TNF-α, whereas IFN-γ had no such effect on CDX2 expression. These, observations indicate that the decreased expression of CDX2 is not a general consequence of inflammation but is more likely a pathway-/signalling-dependent mechanism. Importantly, it points to the fact that this pathway is essential in the understanding of the disease pathogenesis. However, it is important to mention that our *in vitro* analyses are performed in Caco-2 cells which are an immortalised human cancer cell line. Although, the Caco-2 cell line represents the best *in vitro* model it may not represent the exact inflammatory response as seen in the human epithelium.

Therefore, it would be interesting to investigate the TNF-α/CDX2 pathway in primary human IECs. In fact, in collaboration with Department of Gastroenterology, Keio University School of Medicine, Tokyo, the techniques to isolate and long-term culture IECs (organoids) have recently been established in our laboratory at the Department of Gastroenterology, Herlev Hospital. These primary IEC organoids more closely represent the human epithelium than, e.g., Caco-2 or HT29 cells, and is therefore an invaluable new tool for research of more physiological relevance [192]. This technique will also allow us to investigate the impact of other pro-inflammatory cytokines, such as IL-1β and IL-6 on CDX2 expression and characterise their signalling pathways.

Microarray profiles have indicated that patients with active UC treated with IFX have normalised CDX2 expression as compared to non-responsive patients [182,183]. It would therefore be interesting to obtain colonic mucosal biopsies from TNFi naïve patients with active UC prior to and after treatment with TNFi, i.e., IFX, ADA, or GLM to measure mRNA and protein expression levels of CDX2 as well as other differentiation factors and pro-inflammatory markers among responders and non-responders to TNFi.

Our investigations identified for the first time that TNF-α reduced the binding of CDX2 to gene regulatory regions of target genes herein inhibitors of β-catenin. The future course of research in this field should be to investigate whether CDX2 directly affects the activity of β-catenin.

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SUMMARY

Background: High levels of pro-inflammatory cytokines are present in the gut of patients with inflammatory bowel disease (IBD) and have a crucial role in the dysfunction of mucosal homeostasis. *Caudal*-related homeobox transcription factor 2 (CDX2) is an essential regulator of intestinal epithelium homeostasis. When expressed, CDX2 modulates a diverse set of processes, including cell proliferation and differentiation, and cell adhesion and migration. In addition to these critical cellular processes, there is increasing indications (such as involvement in pro-inflammatory pathways and regulation of genes of importance for intestinal homeostasis) linking CDX2 to intestinal inflammation.

Aim: The overall objective of this PhD thesis was to explore the role of CDX2 in the colonic epithelium of patients with

IBD and to investigate the impact of pro-inflammatory pathways on the expression of CDX2 and its target genes.

Methods: Initially, a literature research was conducted to review the importance of CDX2 in epithelial homeostasis and its potential role in mucosal inflammation. Expression of CDX2 was measured in colonic biopsies of patients with IBD, and the effects of pro-inflammatory cytokines and anti-TNF-α treatment (infliximab) were examined in Caco-2 cells. The role of CDX2 in the transcriptional regulation of *MEP1A* and the β-catenin degradation complex genes *APC*, *AXIN2*, and *GSK3β* were investigated in Caco-2 cells. Finally, the intracellular signalling pathways involved in the TNF-α-mediated down-regulation of CDX2 were investigated.

Results: The expression of CDX2 was decreased in the epithelium of patients with ulcerative colitis (UC), and the expression was inversely correlated with the level of TNF-α. The endogenous expression of CDX2 in Caco-2 cells was decreased by TNF-α through NF-κB and p38 MAPK pathways. High levels of TNF-α affected the expression of CDX2 target genes and reduced the binding of CDX2 to gene regulatory regions of these targets in Caco-2 cells. Finally, TNF-α increased the expression of well-known Wnt target genes.

Conclusions: These findings indicate that the CDX2 expression is decreased in UC, and moreover, the studies in Caco-2 cells have shed light on some of the molecular pathways of importance in the inhibitory effect of TNF-α. In addition, these studies have provided novel insight into the CDX2-mediated regulation of genes involved in the β-catenin degradation complex and in response to the pro-inflammatory cytokine, TNF-α. In particular, these findings indicate that CDX2 is involved in the crosstalk between TNF-α and the Wnt signalling pathway, which might be of importance in understanding the link between intestinal inflammation and tumourigenesis.

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