Involvement of CDX2 in the cross talk between TNF-α and Wnt signaling pathway in the colon cancer cell line Caco-2

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Tumor necrosis factor-α (TNF-α) is highly upregulated in inflammation and reduces the expression of the intestinal transcription factor, Caudal-related homeobox transcription factor 2 (CDX2). Wnt/β-catenin signaling is critical for intestinal cell proliferation, but a decreased CDX2 expression has influence on the Wnt signaling-related genes and progression of colorectal cancer. Although several inflammatory signaling pathways, including TNF-α, have been reported to promote Wnt/β-catenin activity and development of cancer, the underlying molecular mechanisms remain unclear. The aim was to investigate the signaling pathways involved in the TNF-α-mediated downregulation of CDX2, and its influence on Wnt/β-catenin signaling components in colon cancer cells. The expression of TNF-α and CDX2 at the invasive front were evaluated by immunohistochemical staining and showed reduced CDX2-positive cells in tumor budding areas with TNF-α expression in the surrounding inflammatory cells. In vitro studies revealed that TNF-α treatment showed a dose-dependent decrease of CDX2 messenger RNA (mRNA) and protein expression in Caco-2 cells. Inhibition of nuclear factor-kappaB or p38 pathways showed that these are involved in the TNF-α-dependent downregulation of CDX2. Furthermore, TNF-α-mediated downregulation of CDX2 was found to significantly decrease the mRNA levels of adenomatous polyposis coli (APC), axis inhibition protein 2 (AXIN2) and glycogen synthase kinase-3 beta (GSK3β), whereas the mRNA levels of Wnt targets were significantly elevated in TNF-α-treated Caco-2 cells. These findings were associated with reduced binding of CDX2 to promoter or enhancer regions of APC, AXIN2 and GSK3β. In conclusion, it was found that TNF-α induces the expression of Wnt signaling components through a downregulation of the CDX2 expression that might have a tumor-promoting effect on colon cancer cells.

Introduction

One of the major signaling pathways involved in the establishment of intestinal homeostasis is the canonical Wnt/β-catenin pathway. Wnt signaling is fundamental in order to maintain the proliferative compartment of the intestinal crypt but a dysregulated Wnt signaling consists of the core proteins, AXIN2, APC, glycogen synthase kinase-3 beta (GSK3β) and casein kinase-1 (33). Hence, it is possible that downregulation of CDX2 expression in the invasive front in CRC contributes to enhanced tumorigenesis by regulating components in the Wnt/β-catenin signaling pathway.

Abbreviations: APC, adenomatous polyposis coli; AXIN2, axis inhibition protein 2; CDX2, Caudal-related homeobox transcription factor 2; ChIP, chromatin immunoprecipitation; CRC, colorectal cancer; ERK1/2, extra-cellular signal-regulated kinase 1/2; GSK3β, glycogen synthase kinase-3 beta; IBD, inflammatory bowel disease; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; NF-κB, nuclear factor-kappaB; qPCR, quantitative real-time PCR; TNF-α, tumor necrosis factor-α; UC, ulcerative colitis.

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1/2 (ERK1/2) might reduce the transcriptional activity of CDX2 (37,38) and downregulate the expression of CDX2 (39) in undifferentiated crypt cells and CRC cells, respectively. Phosphorylation of CDX2 by p38 MAPK accompanies cell differentiation and enhances its transcriptional activity (40). However, the p38-mediated phosphorylation of CDX2 is dependent on the type of stimulus and signaling. Furthermore, a previous study has demonstrated that TNF-α regulates the CDX2 expression through nuclear factor-kappaB (NF-kB) in the colon cancer cell line, HT29 (41). Recently, we have shown that CDX2 expression is decreased in patients with UC and that TNF-α suppresses the expression and function of CDX2 in Caco-2 cells (42); however, the exact molecular mechanism is still unknown.

Although the development of CRC is highly associated to the grade of inflammation and eventually has consequences on the survival rate (43), it is still unclear, which role cytokines released from tumor-associated macrophages and other inflammatory cells have on the solid tumor. As the CDX2 expression is downregulated in colitis and decreased at the invasive front, it is possible that TNF-α-mediated downregulation of CDX2 could be an important cause for metastasis.

In the present study, we have investigated the intracellular signaling pathways involved in the TNF-α-mediated downregulation of CDX2 expression (i.e. NF-κB, p38 and ERK1/2 pathways) in Caco-2 cells. We further examined the influence of TNF-α-mediated downregulation of CDX2 on target genes in the β-catenin degradation complex and in the Wnt signaling pathway. Furthermore, we have investigated the expression of TNF-α and CDX2 by immunostaining carcinoma specimens from CRC patients with tumor budings.

Materials and methods

Patients and tissue samples

Fifty-five rectal specimens with carcinoma were previously obtained from patients undergoing surgical resection at the Department of Pathology, Hvidovre Hospital in 2006. This material has previously been characterized for tumor cell budding (32). Additionally, based on hematoxylin-eosin-stained sections, this material has been characterized according to the extent of inflammation at the invasive margin of the tumor, using a three-tiered scale: mild, moderate and severe inflammation. This study was approved by the Scientific Ethics Committee of the Region Sjaelland, Denmark.

Immunohistochemistry and evaluation of immunostainings

Among the fifty-five formalin-fixed and paraffin-embedded rectal carcinomas previously collected, 10 cases were selected for histological analysis based on the extent of inflammation at the invasive tumor edge [mild inflammation (n = 3), moderate inflammation (n = 3) and severe inflammation (n = 4)] scored semi-quantitatively by two pathologists independently in a blind fashion. Sections were immunostained for CDX2, using anti-human CDX2 (1:300, mouse monoclonal; DAK-CDX2; Dako, Glostrup, Denmark) and for TNF-α, using anti-human TNF-α (1:175, rabbit polyclonal ABB-177; Nordic Biosite, Copenhagen, Denmark).

Negative controls were performed by omission of the primary antibody. The immunohistochemistry was performed as described previously (32). The immunoprofile was assessed at the invasive front of the carcinomas, focusing on zones of budding and zones of non-budding, respectively, by the staining intensity of CDX2 and the proportion of inflammatory cells reactive with TNF-α.

Cell culture and treatment

The human colonic Caco-2 cell line (American Type Culture Collection, Rockville, MD) cells were cultured in monolayers and maintained as described previously (44). For treatment experiments, 1 × 10⁵ cells were seeded in 24-well plates (NUNC Brand; Thermo Fisher, Rochester, NY) and grown to >95% confluence. Cells were then stimulated in medium with or without TNF-α (R&D Systems, Minneapolis, MN) in presence or absence of one of the following chemical inhibitors: tosyl phenylalanyl chloromethyl ketone (NF-κB inhibitor, 100 μM; Sigma–Aldrich, St Louis, MO), SC-409 (the p38 inhibitor, 10 μM; Merck Chemicals, Darmstadt, Germany), FR180204 (ERK inhibitor, 30 μM; Merck Chemicals) or vehicle (0.4% dimethyl sulfox ide; Sigma–Aldrich). In experiments involving treatment with inhibitors, cells were exposed to the inhibitors 1 h prior to addition of TNF-α and subsequently stimulated with TNF-α (10nM) for 24 h.

RNA extraction and quantitative real-time PCR

Total RNA from Caco-2 cells was extracted using the NucleoSpin columns (Macherey-Nagel, Düren, Germany) following the manufacturer’s protocol. A total of 500 ng of each RNA sample was used for complementary DNA synthesis with SuperScript III reverse transcriptase (Invitrogen, Paisley, UK), and all quantitative real-time PCR (qPCR) reactions were performed on a Stratagene Mx3000P thermocycler (Stratagene, La Jolla, CA) using the Maxima SYBR Green qPCR Master Mix (Fisher Scientific, Pittsburgh, PA) according to the manufacturer’s instructions using primers listed in Supplementary Table S1, available at Carcinogenesis Online (32,42). Target gene expressions were normalized to the expression of human Ribosomal Protein Large P0 (RPLP0) serving as reference gene (45), which were amplified in parallel reactions as an internal control.

Protein extraction and immunoblotting

Caco-2 cells were lysed by RIPA lysis buffer (Macherey-Nagel) and proteins were purified according to the manufacturer’s protocol. Proteins from whole cell lysates were then heated at 70°C for 15 min and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4–12% gradient gels). Proteins were transferred to a polyvinylidene difluoride membrane (Expe, San Diego, CA) and incubated with the appropriate primary antibodies overnight at 4°C and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (Dako) followed by enhanced chemiluminescence detection (ProteinSimple, Santa Clara, CA). The primary antibodies were: CDX2 (1:1000; BioGenex Laboratories) and phospho-p65 subunit of NF-κB (1:1000, rabbit polyclonal; Cell Signaling Technology, Danvers, MA). Phosphorylation status of distinct MAPK family members were analyzed by using rabbit monoclonal antibodies directed against p38 and ERK1/2 (p44/42). Both MAPK antibodies were obtained from Cell Signaling Technology and were used at a dilution of 1:1000. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:20 000; mouse monoclonal; Fitzgerald, Concord, MA) was used as loading control.

Chromatin immunoprecipitation assay

Five days after confluence, Caco-2 cells were either stimulated with TNF-α (10nM) or left untreated for 24 h. The cells were cross-linked and sonicated as described previously (35).

Chromatin immunoprecipitation (ChIP) was performed as described previously (42).

Briefly, immunoprecipitation was done in four replicates and performed overnight at 4°C with an antibody specific for either human CDX2 (α-CDX2; BioGenex Laboratories) or an influenza hemagglutinin epitope (rabbit polyclonal α-HA; Santa Cruz Biotechnology, Heidelberg, Germany) used as a negative control. Immunocomplexes were recovered with 50 μl protein A/G beads (Invitrogen, Grand Island, NY). Purified immunoprecipitated DNA and input DNA were analyzed by qPCR. The primers used to amplify the human genomic sequences of APC, AXIN2 and GSK3β at CDX2 target loci were described previously (32). Quantification of the ChIP–DNA was done using the method described by Frank et al. (46).

Statistical methods

Values are presented as medians with interquartile ranges. Groups were compared using the Mann–Whitney U-test. Two-sided P values were considered significant if P < 0.05. Non-parametrical statistical analysis was done using GraphPad Prism version 5.

Results

Immunohistochemical staining of CDX2 and TNF-α expression in patients with CRC

One of the studied carcinomas was mucinous in type, and two additional specimens comprised a minor mucinous component but fulfilled the criteria of usual glandular carcinoma, as did the remaining seven cases. The invasive tumor margin of all cases comprised zones of budding. All but one of the cases comprised additionally non-budding zones. All specimens displayed at least a focal inflammatory reaction (comprising neutrophils, macrophages, lymphocytes and plasma cells in different proportions) at the invasive tumor front. As illustrated in Figure 1A and B, areas of downregulated CDX2 expression were also positive for TNF-α immunostaining. In non-budding zones, CDX2 was characteristically highly expressed and TNF-α expression was absent (Figure 1C and D). Thus, TNF-α expression was observed in the invasive front of the carcinomas which was associated with a decreased CDX2 expression level.

TNF-α decreases the level of CDX2 expression in Caco-2 cells

Caco-2 cells were treated with increasing concentrations of TNF-α (0.1, 1 or 10 nM) for 24 h to investigate the effect of TNF-α on the
Fig. 1. Representative area of the deep invasive front of rectal carcinomas immunostained for CDX2 and for TNF-α, conducted on sequential sections. (A) Tongues of coherent tumor cells (far right and left) surround a central zone with multiple budding cells, several (albeit not all) of which display decreased nuclear CDX2 expression. The latter can be observed in the magnified picture in which two of the three tumor nuclei are only weakly stained for CDX2. (B) The sequential section immunostained for TNF-α demonstrates numerous immunopositive inflammatory cells throughout the budding area. The cytoplasmic localization of TNF-α can readily be appreciated in the magnified section. (C) Illustrates a non-budding zone with high CDX2 expression. (D) The adjacent stroma comprises only few inflammatory components and the TNF-α expression is minimal. The original magnification, (A and B); ×100 and (C and D); ×250.
endogenous CDX2 messenger RNA (mRNA) and protein expression. Treatment with TNF-α significantly reduced the expression of CDX2 mRNA in a dose-dependent manner with maximal reduction observed at 10 nM (P < 0.01) (~50% reduction) (Figure 2A). Similarly, western blot analysis showed that the CDX2 protein abundance was decreased in cells treated with increasing concentrations of TNF-α (Figure 2B). Quantification by densitometry scanning showed an ~60% reduction in CDX2 protein level (P < 0.01) in cells stimulated with 10 nM TNF-α (Figure 2B).

The role of MAPK cascade and transcription factor NF-κB in the TNF-α-mediated downregulation of CDX2 expression in Caco-2 cells

Activation of NF-κB, p38 and ERK1/2 pathways has been demonstrated previously to regulate the expression of CDX2 (37–41). Therefore, the role of these signaling pathways in the TNF-α-mediated downregulation of endogenous CDX2 expression in Caco-2 cells was investigated. The NF-κB inhibitor tosyl phenylalanyl chloromethyl ketone effectively diminished the effect of TNF-α on CDX2 mRNA, indicating that a TNF-α-mediated downregulation of CDX2 mRNA requires NF-κB activation (Figure 3A). Western blot analysis revealed that tosyl phenylalanyl chloromethyl ketone partially reduced the TNF-α-mediated reduction of CDX2 protein expression (Figure 3B). Inhibition of p38 or ERK1/2 with SC-409 or FR180204, respectively, significantly increased the level of CDX2 mRNA (P < 0.05 and P < 0.01, respectively) compared with the vehicle dimethyl sulfoxide but did have only a minor effect on the inhibitory effect of TNF-α on CDX2 mRNA expression (P < 0.05, respectively) (Figure 3A). 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 (Figure 3C). Treatment with FR180204 increased CDX2 protein expression but had no effect on the TNF-α-mediated inhibition of CDX2 protein expression (Figure 3D). 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 (Figure 3B–D). These results provide new insight into the molecular explanation for how TNF-α downregulates the expression of CDX2.

TNF-α reduces the expression and interaction of CDX2 with genes encoding the β-catenin degradation complex

We have recently demonstrated that components of the Wnt signaling pathway are regulated by CDX2 (32). Therefore, we investigated whether a TNF-α-mediated downregulation of CDX2 could affect the endogenous mRNA level of these CDX2 targets; namely APC, AXIN2 and GSK3β. Significant reductions of APC, AXIN2 and GSK3β mRNA levels (P < 0.05, P < 0.01, P < 0.05, respectively) were observed when Caco-2 cells were treated with TNF-α (Figure 4A). Interestingly, the mRNA reduction levels of these targets were almost comparable with the ~50% reduction of CDX2 mRNA in TNF-α-treated (10 nM) Caco-2 cells (see Figure 2).

We have earlier demonstrated that CDX2 binds to upstream enhancer elements in the APC and AXIN2 genes and to the promoter region of GSK3β (32). To investigate the effect of TNF-α on the physical interaction between CDX2 and the gene regulatory regions of APC, AXIN2 and GSK3β, we performed a ChIP assay. Chromatin–protein complexes from untreated and TNF-α-treated Caco-2 cells were immunoprecipitated with a CDX2-specific antibody or a hemagglutinin antibody (negative control). The enrichments of CDX2–DNA complexes were 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 (Figure 4B). These results indicate that reduction of CDX2 expression upon TNF-α stimulation has an influence on regulation of genes in the β-catenin degradation complex.

TNF-α enhances the expression levels of Wnt target genes in Caco-2 cells

Since, CDX2 regulates components of the β-catenin degradation complex and the regulatory function of CDX2 is suppressed by proinflammatory TNF-α, we examined the influence of TNF-α on the expression of Wnt-related target genes via qPCR in Caco-2 cells. Exposure of Caco-2 cells with TNF-α increased the expression of well-known Wnt targets (Figure 5). 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 (Figure 5). These findings demonstrate

Fig. 2. Effect of TNF-α on endogenous expression of CDX2 mRNA and protein levels in Caco-2 cells. (A) Dose-dependent decrease in CDX2 mRNA expression in cells treated for 24 h with increasing concentrations of TNF-α (0.1, 1 or 10 nM). Values are medians with interquartile ranges (n = 6), *P < 0.05, **P < 0.01. (B) Western blot analysis of CDX2 protein levels in TNF-α-treated cells and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as an internal loading control. Densitometric analysis of CDX2 protein level is shown. Data are presented as intensity of the CDX2 protein relative to the GAPDH intensity in the corresponding sample and the protein level in untreated cells was considered 100%. The shown blots represent three independent experiments, *P < 0.05, **P < 0.01. Densitometric analyses were performed using ImageJ software (n = 3).
that Wnt signaling target genes are activated by the proinflammatory cytokine TNF-α.

**Discussion**

The proinflammatory cytokine TNF-α is a critical mediator of inflammatory disorders and is likely to activate signaling pathways involved in tumorigenesis (16). Another key pathway implicated in many types of cancer, including CRC, is the Wnt pathway (2). In particular, mutations in the Wnt/β-catenin pathway are strongly linked to the development of CRC, and an aberrant activity of this pathway is manifested by stabilization of nuclear β-catenin (47). We have recently demonstrated that CDX2 regulates gene expression of components of the β-catenin degradation complex (32), and increasing evidences indicate that CDX2 inhibits the activity of Wnt/β-catenin signaling pathway and thereby probably the aggressiveness of the colorectal tumor (28,29). Moreover, we have just shown that TNF-α suppresses the expression and transcriptional activity of CDX2 in vitro (42). Although several inflammatory signaling pathways have been reported for the promotion of Wnt/β-catenin activity, including TNF-α (48), the underlying molecular mechanisms, however, remain unclear.

In this study, we have determined the expression patterns of TNF-α and CDX2 at the invasive front in CRC, furthermore, we have investigated the mechanism of TNF-α-mediated downregulation of CDX2 and its influence on the expression of Wnt/β-catenin signaling genes in vitro in Caco-2 cells. We have shown that CDX2 expression is downregulated in the invasive front and in tumor buds, which are capable of migrating short distance from the solid tumor in CRC (49). This is in accordance with previous studies demonstrating that transient downregulated CDX2 expression is associated with migration of CRC cells (24,25,27). We also found that TNF-α was expressed in inflammatory cells intermingling the stroma surrounding the invasive front from CRC, which is in concordance with the observation that some tumors are heavily infiltrated by innate immune cells such as macrophages (50). Although inflammation has a protective role, it has become evident that inflammation by innate immune cells can contribute to angiogenesis, invasion and metastasis (51). Furthermore, several studies have suggested a therapeutic targeting of proinflammatory cytokines released by innate and adaptive cells as a promising strategy for the treatment of CRC (52). However, limited studies have investigated the expression of proinflammatory cytokines in the microenvironment surrounding the solid tumor by immunohistochemistry in CRC.

CRC is one of the most serious and life-threatening complications of chronic IBD, but key mediators involved in the link between IBD and development of carcinogenesis is still not fully revealed. In this
effort, Popivanova et al. (53) have previously identified an important role of TNF-α in development of colon cancer. They demonstrated that TNF receptor 1-deficient (TNFR1−/−) mice exposed to dextran sodium sulfate and azoxymethane had reduced inflammation and fewer colitis-associated tumors than wild-type mice. They further demonstrated that wild-type mice treated with anti-TNF-α antibody (etanercept) after administration of azoxymethane and dextran sodium sulfate had a decreased amount of dephosphorylated (i.e. active/nuclear) form of β-catenin as compared with untreated mice (53). Likely, Greten et al. (54) have demonstrated that inactivation of the NF-κB pathway reduces the development of colon cancer induced by azoxymethane and dextran sodium sulfate treatment, which might explain the enhanced NF-κB activity seen in cancer (55). Thus, it is likely that cytokine-triggered activation of NF-κB in inflammatory intestinal epithelial cells may induce and initiate the development of CRC. Indeed, a recent study by Schwitalla et al. (56) has revealed that β-catenin stabilization by NF-κB in a TNF-α-dependent pathway enhances Wnt signaling and induces dedifferentiation of epithelial non-stem cells into tumor-initiating cells.

We have with the present study demonstrated that TNF-α down-regulates the endogenous levels of CDX2 expression, and that the inhibitory effect of TNF-α is mediated by NF-κB and partially by p38 MAPK. Concerning ERK1/2 inhibition, our results are in line with the previous findings by Lemieux et al. (37) and Krueger et al. (39), because inhibition of ERK1/2 increases the endogenous levels of CDX2 mRNA and protein levels. Recently, we have demonstrated that components of the Wnt/β-catenin signaling pathway are regulated by CDX2 and shown that CDX2 interacts with genes in the β-catenin degradation complex (APC, AXIN2 and GSK3β) (32). Here, we found decreased expression of APC and AXIN2 genes in TNF-α-treated cells in accordance with our recent findings in CDX2 knockdown experiments (32). However, we observed that TNF-α treatment reduced the expression of GSK3β in Caco-2 cells, whereas our recent findings showed that CDX2 knockdown increases the expression of GSK3β (32). Moreover, the ChIP assays in Caco-2 cells revealed that TNF-α treatment suppressed the CDX2 binding to the gene regulatory regions of APC, AXIN2 and GSK3β. These results are compatible with previous findings that TNF-α treatment represses the CDX2 expression and its binding affinity to target genes (41,42). Therefore, it is likely that the GSK3β expression is dependent on other factors, which are activated by TNF-α, and these findings indicate that the CDX2 binding is not linked to the expression of GSK3β. Thus, it is plausible to speculate that inflammatory cell infiltrates increase extracellular stimuli by TNF-α to activate NF-κB and to induce epithelial proliferation by suppressing epithelial CDX2 expression and increasing Wnt signaling, thereby causing initiation and development of CRC (56).

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 signaling. Accordingly, we found an increased mRNA expression level of SOX9, cyclin D1, cMYC and HEF1—reported as being direct Wnt pathway target genes (57–60)—in Caco-2 cells exposed to TNF-α.

We have recently reported that CDX2 expression is decreased in inflamed colonic mucosa of patients with UC and are inversely correlated to the mucosal level of TNF-α (42). Moreover, studies have shown that dysregulation of the differentiation system for correct intestinal epithelial cell formation has a crucial role in pathogenesis of UC (61). Interestingly, CDX2 suppression results in goblet cell depletion in UC (62). Thus, given the importance of CDX2 in regulating the expression of...
CDX2 in cross talk between TNF-α and Wnt signaling

Fig. 5. 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). The mRNA levels were normalized to the level of RPLP0 and changes in expression were calculated. Values are represented as medians with interquartile ranges (n = 6), *P < 0.05, **P < 0.01.

Various genes that govern the proliferation and differentiation of epithelial cells (19,63), and that CDX2 is necessary for other epithelial-specific transcription factors to work properly (64), it is likely that during chronic inflammation and cancer. It is also likely that during chronic inflammation, and that CDX2 is necessary for other epithelial-specific transcription factors to work properly. These findings provide novel insight into the molecular regulation of genes involved in the β-catenin degradation complex in response to the proinflammatory cytokine TNF-α, which might be of importance for understanding the link between inflammation and tumorigenesis.

Supplementary material

Supplementary Table S1 can be found at http://carcin.oxfordjournals.org/

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