



Association of a Nkx2-3 polymorphism with Crohn's disease and expression of Nkx2-3 is up-regulated in B cell lines and intestinal tissues with Crohn's disease

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Abstract

Aim: To replicate the association of Nkx2-3 rs10883365 SNP with Crohn's disease in patients from a familial IBD registry from the central Pennsylvania area and study mRNA and protein expression of Nkx2-3 in CD patients.

Materials and methods: We genotyped the Nkx2-3 rs10883365 SNP in 75 CD patients, 137 non-CD family members and 118 unrelated healthy controls from EBV-transformed B cell lines of a familial IBD registry in central Pennsylvania. mRNA and protein expression levels of Nkx2-3 were measured by RT-PCR and Western blot, respectively.

Results: rs10883365 was found to be associated with CD. A significant difference between the homozygous variant genotype (GG) compared to the wild type sequence (AA) was observed between CD and individuals without IBD, including both non-IBD family members from the familial IBD registry and unrelated healthy controls. However, there was not a significant difference between CD and non-IBD related family members. mRNA and protein expression levels of Nkx2-3 were increased in CD compared with non-CD sibling and healthy controls. A total of 16 sibling pairs were examined, and the mRNA and protein expression levels of Nkx2-3 from 12 of the sibling pairs (75%) were increased in the CD individual compared with the non-CD sibling. mRNA expression levels of Nkx2-3 were increased in diseased tissues compared with adjacent normal tissues in 7 of 9 patients (77.8%).

Conclusions: Nkx2-3 is genetically associated with CD and is up-regulated in CD, suggesting that Nkx2-3 is involved in the pathogenesis of CD.

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1. Introduction

The inflammatory bowel diseases (IBD) are comprised of two major forms, Crohn's disease (CD) and ulcerative colitis (UC). They are chronic, relapsing inflammatory disorders of the gastrointestinal tract. Experimental and clinical data suggest that the induction and pathogenesis of the diseases is a multifactorial process involving interactions among genetic, immune, and environmental factors.

Strong familial aggregation, twin studies, and established genetic associations attest to the important role of genetics in IBD pathogenesis.¹ A combination of genome-wide linkage, candidate gene and targeted association mapping studies has been successful in the identification of Crohn's disease-associated genetic variants in the CARD15 and IBD5 locus, however, these explain only a relatively small fraction of the heritability found in Crohn's disease.^{2–5} In order to identify genetic factors influencing the risk of IBD, several groups recently have performed genome-wide association studies (GWAs) and genotyped a large number of single-nucleotide polymorphisms (SNPs) throughout the genome, and identified more than 32 IBD susceptibility loci, including Nkx2-3.^{6–11} Nkx2-3 is a member of the Nkx family of homeobox transcription factors. Homeobox genes have long been studied as determinants of pattern and lineage in multicellular organisms.^{12,13} During development, Nkx2-3 is expressed in midgut and hindgut mesoderm and spleen, as well as in pharyngeal endoderm.^{14–16} Analysis of Nkx2-3-deficient mice has revealed a critical role for this homeobox transcription factor in spleen development and organization,^{17,18} and in establishing the correct environment for normal B cell development and T cell dependent immune response.¹⁹ Nkx2-3 is also expressed in capillary endothelial cells within the lamina propria and submucosa of the intestine, where it is required for expression of the lymphocyte adhesion molecule MAdCAM-1.¹⁷ Abnormal expression of Nkx2-3 may alter gut migration of antigen-responsive lymphocytes and thus influence the intestinal inflammatory response.

Although Nkx2-3 is associated with IBD, its exact role in IBD pathogenesis is unclear. This study aimed to: (1) replicate the association of Nkx2-3 rs10883365 SNP with CD in patients from a familial IBD registry from the central Pennsylvania

area; (2) study mRNA and protein expression of Nkx2-3 in EBV-transformed B cell lines from the familial IBD registry; and (3) study mRNA expression of Nkx2-3 in intestinal tissues from CD patients.

2. Materials and methods

2.1. Establishment of Epstein–Barr virus-transformed B cell lines

EBV was obtained from a cotton-top tamarin EBV-transformed lymphocyte cell line (B95-8) provided by the American Type Culture Collection (ATCC) for transformation of human B cells. Using standard procedures, EBV-B cell lines were established for individual family members entered into a central Pennsylvania IBD patient registry.²⁰ Families were regarded informative in terms of genetic analysis if they included at least two generations with two parents and at least two children available at the time of recruitment with at least two individuals affected with disease. Only such informative families were studied here. This project received approval from the Institutional Review Board of the Milton S. Hershey Medical Center. EBV-transformed B cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO₂.

2.2. Patients and intestinal tissue samples

Nine adult patients undergoing surgery at the Penn State Hershey Medical Center for a diagnosis of CD participated in this study. An informed consent was signed by the patients before surgery. Macroscopically normal areas of intestine and areas of intestine with obvious disease were obtained from various regions of the colon, proximal small bowel and ileum after resection. The intestinal tissues were immediately submerged with RNAlater Soln (Ambion, CA, USA) at 4 °C overnight. Then they were stored frozen at –80 °C until total RNA extraction. The protocol for tissue collection was approved by the institutional review board at the Penn State Hershey Medical Center.

Table 1 Genotype of Nkx2-3 rs10883365 in Crohn's disease.

Genotype	Crohn's diseases	Total non-CD control	OR	95% CI	p-value
GG	24 (32%)	62 (24.3%)	1.0972	0.9477–1.2702	0.0483
AG	37 (50.3%)	119 (46.7%)			
AA	14 (18.7%)	74 (29%)			
Genotype	Crohn's diseases	Healthy control	OR	95% CI	p-value
GG	24 (32%)	26 (22%)	1.2372	0.9231–1.6583	0.0412
AG	37 (50.3%)	54 (45.8%)			
AA	14 (18.7%)	38 (32.2%)			
Genotype	Crohn's diseases	Non-CD family members	OR	95% CI	p-value
GG	24 (32%)	36 (26.3%)	1.1075	0.8751–1.4015	0.085
AG	37 (50.3%)	65 (47.4%)			
AA	14 (18.7%)	36 (26.3%)			

Table 2 Sixteen sibling pairs and 2 healthy controls from EBV-transformed B cell lines.

A. Characterization of 16 sibling pairs and 2 healthy control			
	Gender		Average ages
	Male	Female	
Crohn's disease	8	8	42.9
Non-IBD sibling	9	7	42.8
Healthy	1	1	36

B. Genotype of Nkx2-3 rs10883365 in 16 sibling pairs		
Genotype	Crohn's disease	Non-IBD sibling
GG	6 (37.5%)	5 (31.25%)
AG	6 (37.5%)	8 (50%)
AA	4 (25%)	3 (18.75%)

Genotype of rs10883365 is the same between CD and non-CD in 9 sibling pairs: AA (1 sibling pair), AG (5 sibling pairs), GG (3 sibling pairs).

2.3. DNA extraction and Genotyping for Nkx2-3

DNA was extracted from EBV-transformed B cell lines using QIAamp DNA Mini Kit according to the manufacturer's instruction (QIAGEN Sciences, MD, USA). SNP rs10883365 genotyping was performed using SNPlex™ Genotyping System as previously described.⁹ In brief, 100 ng of genomic DNA was dried overnight in TwinTec 384well plates (Eppendorf, Hamburg, Germany) at room temperature. Genotyping process was performed using SNPlex™ chemistry (Applied Biosystems, Foster City, USA) on an automated platform with TECAN Freedom EVO and 384well TEMO liquid handling robots (TECAN, Männedorf, Switzerland). Genotype assignments were confirmed by visual inspection with the Genemapper® software v4.0 (Applied Biosystems).

2.4. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from EBV-transformed B cell lines and intestine tissues using RNeasy mini Kit according to the manufacturer's instruction (QIAGEN Sciences, MD, USA). cDNA

was synthesized from 1.5 µg of total RNA using Superscript III 1st Strand Synthesis Kit (Invitrogen, CA, USA).

Primers for human Nkx2-3 (Forward 5'-CCACCCCTTCTCAG-TCAAA-3' and Reverse 5'-CTGCGGCTAGTGAGTTCAA-3') and GAPDH (Forward 5'-TGATGACATCAAGAAGGTGGTGAAG-3' and Reverse 5'-TCCTTGGAGCCATGTGGGCCAT-3') were designed using Primer3 software. PCR amplifications were performed at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 45 s for 38 cycles (Nkx2-3) and 28 cycles (GAPDH) respectively, followed by final extension at 72 °C for 7 min. The PCR product for Nkx2-3 is

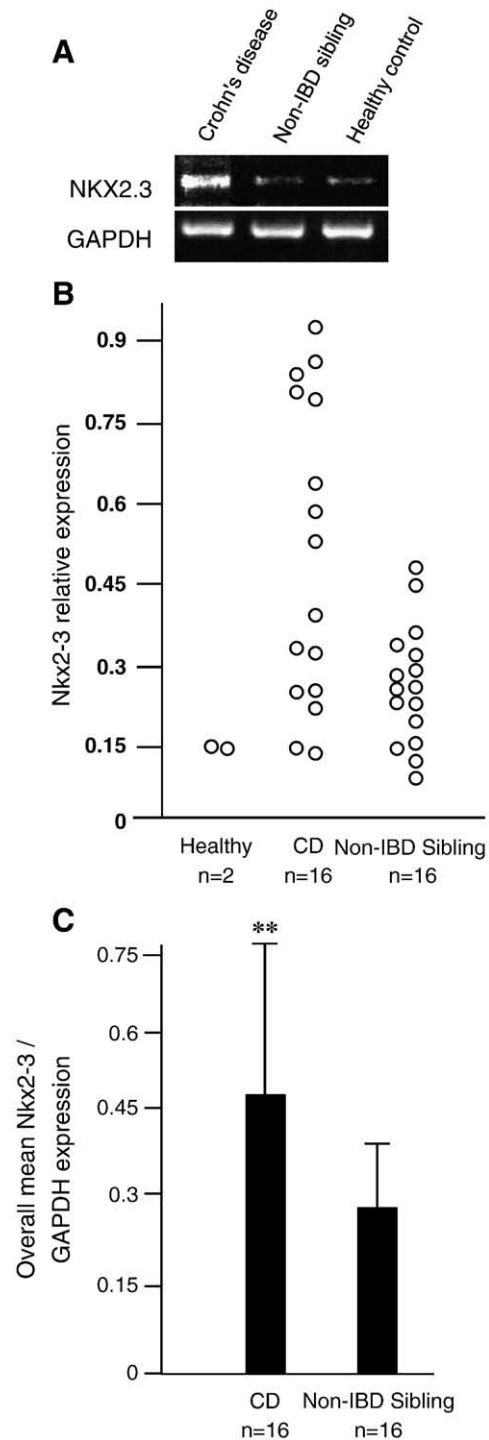


Figure 1 Nkx2-3 mRNA expression is up-regulated in EBV-transformed B cells from CD patients. (A) Representative RT-PCR result: mRNA expression level of Nkx2-3 was increased in CD compared with non-CD sibling and healthy control. GAPDH expression served as a control. (B) Relative mRNA expression levels of Nkx2-3 of 16 sibling pairs and 2 healthy controls. The RT-PCR products bands on the photograph were scanned by densitometry. The relative mRNA expression level was expressed as Nkx2-3 compared to GAPDH. (C) Comparison of Nkx2-3 mRNA expression levels between CD patient and non-CD sibling in 16 sibling pairs. The values represent the fold change in densitometry data as described in (B). Between the Crohn's disease group and non-CD sibling group, the mRNA expression level of Nkx2-3 is significantly different (** $p < 0.01$). Data are presented as the means \pm standard errors.

210 bp and GAPDH is 236 bp. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

2.5. Western blotting

Western blotting was performed as described previously.²¹ Briefly, EBV-transformed B cell lines were lysed with RIPA buffer (25 mM Tris-HCl, PH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and protease inhibitor cocktail (Roche, Germany). Protein (30 µg/lane) was separated using SDS-15% PAGE and transferred onto nitrocellulose membranes. Nitrocellulose membranes were blocked by 5% dry milk and probed overnight at 4 °C with anti-Nkx2-3 polyclonal antibody (Abcam, Cambridge, MA, USA). After Nkx2-3 protein detection, the membrane was stripped by stripping buffer (Restore Western blot Stripping buffer, Thermo Scientific, IL, USA) for 30 min at 37 °C. After stripping, the membrane was washed, blocked, and reprobbed with anti-Actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described above.

2.6. Statistical analyses

Cochrn–Mantel–Haenszel Statistics was used for genotype analysis. To evaluate associations with different traits two-sided *p*-values were calculated according to Fisher using contingency tables. Odds ratios are given with 95% confidence intervals. *p*<0.05 was considered significant.

For RT-PCR and Western Blotting, data are presented as the mean±standard error. *p*<0.05 was considered significant.

3. Results

3.1. Genotype analysis of Nkx2-3 SNP rs10883365

Recent genome-wide association studies have shown that rs10883365, located in the upstream region of Nkx2-3 (about 5000 bp from transcription start region), has been associated with both European and Japanese Crohn's Disease populations.^{6–11} We analyzed the rs10883365 variation in 75 Crohn's disease patients, 137 non-CD family members and 118 unrelated healthy controls from EBV-transformed B cell lines of a familial IBD registry in central Pennsylvania (Table 1).

As shown in Table 1, rs10883365 was found to be significantly associated with CD. A significant difference between the homozygous variant genotype (GG) compared to the wild type sequence (AA) was observed between CD and all individuals without IBD, including both non-IBD family members from the familial IBD registry and unrelated healthy controls: OR=1.0972 (0.9477–1.2702), *p*=0.0483.

Family history is a significant risk factor for IBD. We further divided the individuals without IBD described above into two groups: non-IBD family members and unrelated healthy individuals. As shown in Table 1, a significant difference between the homozygous variant genotype (GG) compared and the wild type sequence (AA) was detected between CD and unrelated healthy controls: OR=1.2372 (0.9231–1.6583), *p*=0.0412. However, there was not a significant difference between the homozygous variant genotype (GG) compared to the wild type sequence (AA) between CD and non-IBD family members: OR=1.1075

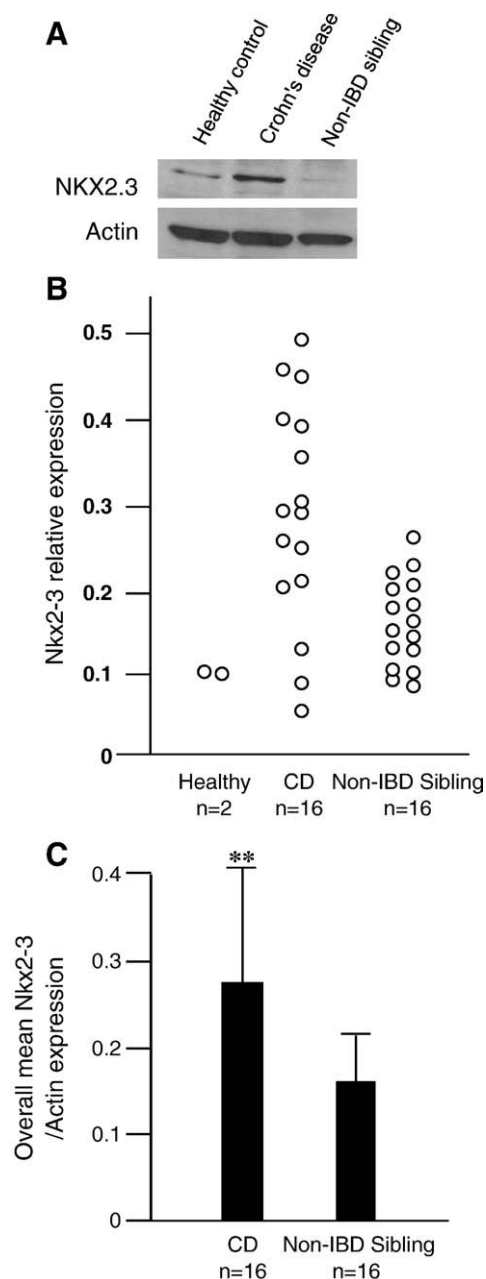


Figure 2 Nkx2-3 protein expression level is up-regulated in EBV-transformed B cells from CD patients. (A) Representative Western blot result: protein expression level of Nkx2-3 was increased in CD compared with non-CD sibling and healthy control. Actin expression served as a control. (B) Relative protein expression levels of Nkx2-3 of 16 sibling pairs and 2 healthy controls. The western blot bands on the photograph were scanned by densitometry. The relative protein expression level was expressed as Nkx2-3 compared to Actin. (C) Comparison of Nkx2-3 protein expression levels between CD patient and non-CD sibling in 16 sibling pairs. The values represent the fold change in densitometry data as described in (B). Between the Crohn's disease group and non-CD sibling group, the protein expression level of Nkx2-3 is significantly different (***p*<0.01). Data are presented as the means±standard errors.

(0.8571–1.4015), $p=0.085$. This result indicates that rs10883365 is associated with CD in patients from this familial IBD registry in central Pennsylvania and is also present in related, but undiseased family members.

3.2. Expression of Nkx2-3 in EBV-transformed B cell lines

Expression of Nkx2-3 has not been previously studied in IBD. We examined the mRNA and protein expression levels of Nkx2-3 in 16 sibling pairs (one diseased and one healthy) and 2 healthy controls from EBV-transformed B cell lines in a CD family registry (Table 2A). As shown in Table 2B, overall, there was no obvious difference in rs10883365 genotype in the 16 sibling pairs: 6 CD patients were of the homozygous genotype (GG) and 5 non-IBD siblings had the homozygous genotype (GG). Moreover, the rs10883365 genotype was the same between the CD and non-IBD sibling in 9 sibling pairs. However, as shown in Fig. 1A and B, the mRNA expression level of Nkx2-3 was increased in the CD individuals compared with the non-CD sibling and healthy controls. A total of 16 sibling pairs were examined, and mRNA expression levels of Nkx2-3 from 12 sibling pairs (75%) were increased in the CD individual compared with the non-CD sibling. The overall mean Nkx2-3 / GAPDH expression values are shown in Fig. 1C: 0.489 ± 0.276 (CD, $n=16$), vs. 0.27 ± 0.12 (non-CD sibling, $n=16$). mRNA Nkx2-3 expression was significantly increased in CD by 1.80-fold compared to non-CD sibling ($p < 0.01$).

We further examined the protein expression levels of Nkx2-3 in the sibling pairs. As shown in Fig. 2A and B, protein expression level of Nkx2-3 was increased in CD compared with non-CD siblings and healthy controls. A total of 16 sibling pairs were examined, and protein expression levels of Nkx2-3 from 12 sibling pairs (75%) were increased in the CD compared with the non-CD sibling. The overall mean Nkx2-3 / Actin expression values are shown in Fig. 2C: 0.287 ± 0.13 (CD, $n=16$), vs. 0.156 ± 0.56 (non-CD sibling, $n=16$). Protein Nkx2-3 expression was significantly increased in CD by 1.84-fold compared to the non-CD sibling ($p < 0.01$).

3.3. Expression of Nkx2-3 in intestinal tissues

Next we examined the mRNA expression of Nkx2-3 in intestinal tissues. Diseased intestinal and adjacent non-diseased samples obtained from 9 Crohn's disease patients were used for this study (Table 3). As shown in Fig. 3A and B, the mRNA expression

Table 3 Intestinal segments of 9 patients in this study.

Patients	Diseased tissues	Healthy tissues
1	Rectum	Left/Sigmoid colon
2	Small bowel	Small bowel
3	Small bowel	Small bowel
4	Terminal ileum	Terminal ileum
5	Right colon	Cecum
6	Left/Sigmoid colon	Cecum
7	Right colon	Right colon
8	Terminal ileum	Terminal ileum
9	Terminal ileum	Terminal ileum

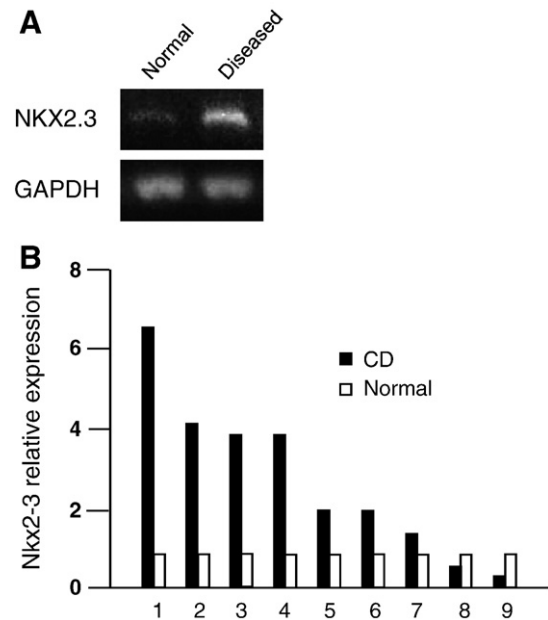


Figure 3 Nkx2-3 mRNA expression level is up-regulated in intestine tissues from Crohn's disease. (A) Representative RT-PCR result of one CD patient: mRNA expression level of Nkx2-3 was increased in diseased tissue compared with adjacent normal tissue. GAPDH expression served as a control. (B) mRNA expression levels of Nkx2-3 in 9 Crohn's disease patients. The RT-PCR products bands on the photograph were scanned by densitometry, the ratios of Nkx2-3 to GAPDH were calculated. For every patient, the ratio of Nkx2-3 to GAPDH with normal tissue was arbitrarily set as 1. The mRNA expression levels of Nkx2-3 were increased in diseased tissues compared with adjacent non-diseased tissues in 7 of 9 patients (77.8%).

levels of Nkx2-3 were increased in diseased tissues compared with adjacent normal tissues in 7 of 9 patients (77.8%).

4. Discussion

In this study we replicated the association of rs10883365 with CD in a population of familial IBD patients from the central Pennsylvania area. We also observed that expression of Nkx2-3 was increased in CD both in EBV-transformed B cell lines and intestinal tissues.

SNP rs10883365 is located in the upstream region of Nkx2-3, about 5000 bp from the transcription start site. Its role in Nkx2-3 gene regulation and in IBD pathogenesis is unknown. Nkx2-3 is a transcription factor and Nkx2-3-deficient mice show severe defects of gut development, primarily in the epithelium of the small intestine.²² In addition, the lymphoid organs of these mice, including the spleen and Peyer' patches, have abnormal tissue architecture and abnormalities in the migration and segregation of B and T cells.¹⁸

To investigate the mechanism of Nkx2-3 in IBD pathogenesis, we studied both mRNA and protein expression of this gene. Expression levels of Nkx2-3 have not been previously investigated in CD. Using 16 sibling pairs of CD from EBV-transformed B cell lines, we showed that mRNA and protein expression levels were significantly increased in CD patients compared with their non-CD sibling controls. To confirm our observations in B cell

lines, we examined mRNA expression levels of Nkx2-3 in diseased tissues from 9 CD patients, and the expression levels of Nkx2-3 were significantly increased in diseased tissues compared with adjacent healthy tissues in 7 patients (77.8%). Among the 16 sibling pairs, there was no obvious difference in the rs10883365 genotype. Moreover, the rs10883365 genotype was the same between the CD and the non-IBD siblings in 9 out of the 16 sibling pairs. But the expression levels of Nkx2-3 overall were still significantly increased in the CD group compared with the non-CD sibling group.

As Nkx2-3 is a transcription factor, its up-regulation may affect genes it can regulate, some of which may be involved in the inflammation seen in IBD. Published data indicate that Nkx2-3 controls expression of the leukocyte homing coreceptor, MAdCAM-1, which is involved in migration and segregation of B and T cells.^{17,18} Expression of MAdCAM-1 in the adult is restricted to mucosal tissues and has been shown to be dramatically up-regulated within focal sites of intestinal inflammation in both animal models of IBD²³ and in human tissue samples from patients with CD and ulcerative colitis (UC).^{24,25} Our unpublished data show that Nkx2-3 and MAdCAM-1 have similar expression patterns in EBV-transformed B cells. This leads us to speculate that Nkx2-3 could affect MAdCAM-1 expression and then be involved in development of IBD. However, other genes, yet to be defined, regulated by Nkx2-3 may also play a role.

Several features of the defective splenic microarchitecture seen in Nkx2-3 mutant mice²³ resemble those found in mice lacking components of the signaling pathways for the TNF family of cytokines, including lymphotoxin (LT).^{26,27} TNF- α is a potent proinflammatory cytokine, which plays a central role in inflammation and immunity.²⁸ Many studies have shown the important role of TNF- α in the pathophysiology of Crohn's disease. LT/TNF- α signaling has also been shown to up-regulate MAdCAM-1 expression on endothelial cells *in vitro* and *in vivo*.^{29,30} It is possible that the NKX2-3/Nkx2-3 regulated genes and the TNF alpha cytokine pathway cross regulate each other in IBD through their common ability to affect adhesion molecule expression at the intestinal level.

The exact functional role that Nkx2-3 might play in the etiology of intestinal inflammation and IBD remains to be fully defined. Identification of additional target genes of Nkx2-3 is currently being investigated in our laboratory. As a whole, these data suggest Nkx2-3 may have an important role in CD and may represent a potential diagnostic and therapeutic target for IBD.

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All authors participated and take public responsibility for the content of this article. W. Yu, W.A. Koltun and Z.W. Lin designed and performed this study. W. Yu, Z.W. Lin and W.A. Koltun drafted the manuscript. T.Y. Li performed statistical

analysis. S. Schreiber designed the genotype analysis. A.A. Kelly, J.P. Hegaty, L.S. Poritz and Y.H. Wang prepared clinical samples and collected clinical data. Authors had no writing assistance. All authors read and approved the final manuscript.

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