Nkx2-3—A Slippery Slope From Development Through Inflammation Toward Hematopoietic Malignancies

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ABSTRACT: The development of peripheral lymphoid tissues from the mesoderm is the result of a complex convergence combining lymphohematopoietic differentiation with the local specification of nonhematopoietic mesenchymal components. Although the various transcriptional regulators with fate-determining effects in diversifying the mobile leukocyte subsets have been thoroughly studied and identified, the tissue-specific determinants promoting the regional differentiation of resident mesenchyme are less understood. Of these factors, various members of the NK-class Nkx paralogues have emerged as key regulators for the organogenesis of spleen and mucosal lymphoid tissues, and recent data have also indicated their involvement in various pathological events, including gut inflammation and hematopoietic malignancies. Here, we summarize available data on the roles of Nkx2-3 in lymphoid tissue development and discuss its possible value as a developmental marker and disease-associated pathogenic trait.

KEYWORDS: Nkx2-3, lymphoid organs, lymphoma, inflammation

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Introduction: Roles of Nkx Family Members in Cellular Differentiation and Organogenesis—A General Overview

The development of complex organisms such as the mammalian body requires a highly regulated coordination of gene expression. Homeobox genes containing helix-turn-helix DNA-binding motif first described in *Drosophila* are master regulators of developmental processes such as mesodermal patterning. The largest group of homeobox genes comprises the HOX (including the extended HOX) class of transcription factors, which may be phylogenetically related to the NK class of homeobox genes that constitutes the second largest group of homeobox-encoding genes. In *Drosophila*, there are 2 classes of NK genes, the NK-1 class containing the NK-1 family and the NK-2 class with the highly related NK-2, NK-3, and NK-4 families. Two NK-2 class genes, namely, *tinman* and *bagpipe*, are responsible for dorsal heart tube formation, mesodermal differentiation, and development of gut musculature, respectively.[1] Vertebrate homologues of ancient NK-class homeobox containing genes were identified on their conserved tyrosine residues.[2,3]

All NK-2 members are responsible for the development of a certain organ or tissue, and most of them share an overlapping expression pattern. They are able to activate signaling pathways responsible for cell differentiation, migration, and maturation, thus their activity is essential to the formation and maintenance of a normally structured and functioning organism.[4]

Regulation through Nkx transcription factors encoded by NK-2 genes is a well-balanced system, where every small change can lead to severe alterations. This complexity is well illustrated by the multiple roles of the NK-2 class members. Nkx2.1 and Nkx2.8 are responsible for lung epithelial development and surfactant production, whereas Nkx2.1 is also crucial in the development of thyroid and pituitary glands.[5] Alterations in the *Nkx2.1* gene can cause neurological defects, congenital hypothyroidism, and lung malformations in humans.[6] In addition, *Nkx2.1* also acts as a proto-oncogene in lung tumors,[7] and it is overexpressed in lung adenocarcinoma and small cell lung cancer.[8] Due to its tissue specificity, it can be used for the identification of metastatic cells of lung epithelial origin.[9] Furthermore, Nkx2.1 and Nkx2.8 also have a prognostic value, as their upregulation in human lung squamous carcinoma cells resulted in resistance to cisplatin, a chemotherapeutic agent.[10,11]

Nkx2.2 and Nkx2.9 share an overlapping expression pattern in neural progenitors. They are necessary for the development of spinal cord V3 interneurons and hindbrain visceral motor neurons, as the spinal cords of mice lacking these factors have

reduced number of V3 neurons and expanded motor neurons.[12]

Another important factor is Nkx2.5, an early myogenic marker detected in mouse embryos from embryonic day 7.5 (E7.5), which is responsible for the development of the atrioventricular node and the myocardium. Nkx2.5 is responsible for cardiac looping and the expression of several other transcription factors essential for heart development, angiogenesis, and hematopoiesis, thus its mutation causes an arrest in heart development and embryonic lethality in mice.[13,14] In humans, mutations of *Nkx2.5*, such as a single-nucleotide deletion that was correlated with congenital heart disease, can cause atrial septal defect and sudden cardiac death.[15] Furthermore, *Nkx2.6* has a similar role in heart development; however, its role is less important because *Nkx2.6* null mice are viable due to the compensatory effect of Nkx2.5. This compensation is probably related to the ectopic expression of Nkx2.5 in the lateral pharynx where normally only Nkx2.6 is expressed.[16]

Besides the nervous system, the vascular system and airways, combinatorial expression of Nkx factors can also be observed in abdominal visceral organs, especially in the stomach (Nkx2.5 and Nkx2.6) and spleen (Nkx2.5 and Nkx2.3).

Although the various NK-2 homologues have different sequences reflecting different functions, they display overlapping homeodomain structure and DNA-sequence specificities as defined by its first helical region and carboxy-terminal region and often overlapping expression patterns. In contrast to most of the NK class members which bind DNA with TAAT core sequences,[17,18] homeoproteins of the NK-2 class bind DNA with CAAG core sequences.[19,20] The specific overlapping or distinct expression and DNA-binding specificities of these Nkx factors create a special transcriptional setting, which is essential in development, differentiation, and adult tissue patterning in an organ-specific manner, collectively referred to as "Nkx code."[1]

Roles of Nkx Family Members in the Formation of Lymphoid Tissues and Intestinal Inflammation

Nkx family members also play crucial roles in lymphoid organ development. The spleen, the largest peripheral lymphoid organ, develops following signals originating from the splanchnic mesodermal plate (SMP) during embryonic development,[21] a transient structure expressing Nkx3-2 (also known as Bapx1). The Nkx3-2–producing SMP has an essential role in the formation of left-right asymmetry[21] and the development of spleen from E10-10.5. The absence of this factor causes asplenia and disturbed pyloric sphincter formation due to impairments of the visceral mesoderm. As SMP formation is permitted in the absence of Nkx3-2, it is likely that this factor is not necessary for the appearance of SMP but is required for its maturation to provide further factors promoting spleen development.

Another Nkx member, *Nkx2-5*, is also expressed in the developing spleen and its absence also leads to asplenia.[22] Both Nkx3-2 and Nkx2-5 can serve as early markers of splenic development. Nkx2-5 is expressed in specified splenic mesenchymal cells which will form the splenic anlage.[23] Among these mesenchymal cells, we can find lymphoid tissue organizer cells that serve as stromal precursors of secondary lymphoid organs.[24] According to Castagnaro et al, all splenic stromal cells (fibroblastic reticular cells, marginal reticular cells, follicular dendritic cells [FDCs], and mural cells) derived from Nkx2-5$^+$Islet1$^+$IL-7$^+$ mesenchymal precursors. Interestingly, this derivation is spleen specific, as Nkx2-5 is not involved in the stromal development of mesenteric lymph nodes and Peyer's patches.[25]

In contrast to Nkx2-5 and Nkx3-2 which take part only in spleen development among peripheral lymphoid organs, Nkx2-3 is involved in the formation of other lymphoid tissues as well. Nkx2-3 is expressed in the spleen, midgut, hindgut, and pharyngeal endoderm.[26] Nkx2-3 is crucial for the formation of visceral mesoderm which gives rise to several cell types, such as vascular and intestinal smooth muscle cells, endothelial cells involved in leukocyte traffic, and stromal cells of secondary lymphoid organs.[23] The main effect of Nkx2-3 is the expression of the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in endothelial cells. MAdCAM-1 has a crucial role in lymphocyte homing to mucosal tissues by binding integrin α4β7 and L-selectin leukocyte homing receptors.[27]

Nkx2-3 deficiency in mice results in atrophic disorganized spleen, fewer and smaller Peyer's patches with altered endothelial addressin expression, enlarged and disorganized colonic crypts, abnormal villus formation, and altered lymphocyte homing.[28] In the spleen, the possible target cells affected by Nkx2-3 deficiency are the red pulp (RP) sinus endothelial cells, as in mice lacking Nkx2-3, the venous sinus network identifiable by IBL-9/2 rat mAb[29] is completely missing. The absence of Nkx2-3 also disrupts the architecture of marginal sinus, including the organization of marginal zone (MZ) macrophage subpopulations and MAdCAM-1–positive marginal sinus-lining endothelial cells, similar to the deficiency of lymphotoxin β receptor (LTβR); however, the RP vasculature is unperturbed in the absence of LTβR.[30] In human spleen, the presence of RP venous sinuses lined by CD31$^+$/CD34$^-$/vWF$^+$ endothelial cells that express Nkx2-3 supports the involvement of Nkx2-3 in the regional specification of endothelium.[31] Furthermore, the absence of Nkx2-3 in mice causes ectopic differentiation of splenic vessels into lymph node like high endothelial venules (HEVs) displaying PNAd homing addressin in an LTβR-dependent process.[32] Similarly, the constitutive activation of the noncanonical nuclear factor κB pathway mediating LTβR signaling in *p100*$^{-/-}$/p52knock-in mice also resulted in ectopic PNAd-positive splenic HEVs; however, these endothelial cells coexpressed both MAdCAM-1 and PNAd,[33] whereas the HEVs in adult Nkx2-3–deficient mice only produced PNAd addressin.[32] Interestingly, Nkx2-3 may also affect the vascular commitment of spleen at a very early stage, as its absence also induced the expression of sac-like structures within the spleen lined by endothelial cells

displaying LYVE-1 hyaluronan receptor, characteristic for lymphatic endothelia.[34] In summary, although some features of the absence of Nkx2-3 are reminiscent of the consequences of LTβR inactivation, the "gradient" of splenic tissue alterations manifests in an opposite directionality—in *Nkx2-3*$^{-/-}$ mice, the most severe splenic defect affects the RP, with somewhat lesser defects of white pulp and follicles (including preserved FDCs), whereas in *Ltbr*$^{-/-}$ mice, the RP appears intact, but the white pulp and follicles (lacking FDCs) are severely perturbed. The MZ is affected in both conditions. Furthermore, the formation of Peyer's patches in Nkx2-3–deficient mice is partially blocked,[35] and in the mucosal HEVs, the MAdCAM-1 addressin is also replaced by PNAd.[36]

Microvascular endothelial cells are important mediators of intestinal homeostasis and inflammation. Activation of this endothelial layer by bacterial and other agents results in the upregulation of adhesion molecules and chemokines necessary for the vascular attachment and migration of leukocytes. Altered expression of endothelial markers such as Nkx2-3 and its potential target MAdCAM-1 can cause a perturbation in leukocyte traffic and inflammatory response. Consequently, the development of inflammatory bowel diseases (IBD) such as Crohn disease (CD) and ulcerative colitis (UC) can be linked to these endothelial factors.[37,38] In the past few years, several genome-wide association studies have demonstrated an association between altered expression of Nkx2-3 and IBDs. According to Xiao et al, single-nucleotide polymorphisms of *Nkx2-3* (namely, rs10883365 and rs1190140) are associated with CD and UC. Polymorphism rs10883365 may contribute significantly to the emergence of both CD and UC, whereas occurrence of the T allele of rs1190140 can increase the risk of CD.[39]

Increased expression of Nkx2-3 at both RNA and protein level was also demonstrated in intestinal samples of patients with CD.[38] Connor et al[40] reported that elevated Nkx2-3 level may lead to the upregulation of MAdCAM-1 at inflammatory sites. Tumor necrosis factor α can further augment adhesion molecule expression in the intestines and it may crossregulate the genes affected by Nkx2-3.[38]

In other studies, 125 Nkx2-3–regulated genes were identified in an Nkx2-3 knockdown B-cell line using genome-wide gene expression microarray analysis. This cell line was originated from a patient with CD and showed downregulation of 33 and upregulation of 92 genes following Nkx2-3 knockdown.[41] In this study, a comprehensive list of inflammation-associated genes and their subpathways that are regulated by Nkx2-3 was also provided. According to these findings, Nkx2-3 knockdown caused downregulation of CXCR7 and upregulation of CXCL1, CCL22, and CXCL10 chemokines, hence affecting the chemotactic activities in immune responses.[41] Decreased Nkx2-3 levels also influenced the MEF2/KLF2 pathway, which has an important role in the maintenance of microvascular endothelial balance and induction of inflammation.[42] However, KLF2 also regulates the vasoactive peptide endothelin-1 (END-1), which may have role in the development of IBD. END-1 expression negatively correlates with Nkx2-3 as it was downregulated in the intestinal tissue of patients with UC and CD.[38]

Recently, a positive feedback loop in the expression of AOC3 (amine oxidase, copper containing 3, a marker expressed on cell surface, thus enabling sorting of viable cells) and Nkx2-3 was observed both in human myofibroblast cell lines and primary cultures as colorectal epithelial stem cell niche components. Intriguingly, Nkx2-3 expression not only distinguished intestinal myofibroblasts from skin fibroblasts but it also turned out to be a determinant in the preservation of myofibroblast identity, presumably involved in regulating colonic stem cell niche.[43] This was further proven using small interfering RNA–mediated knockdown of Nkx2-3 in myofibroblast lines, inducing cells to acquire a fibroblast-like genetic profile. The exact connection between the Nkx2.3^{+} myofibroblasts and the recently discovered CD34^{+} gp38^{+} nonmyofibroblastic mesenchymal cells that create a niche for intestinal epithelial stem cells[44] remains to be elucidated.

The lineage-specific expression of Nkx2-3 has not been formally defined yet either in humans or in mice. Using a recently developed and validated immunohistochemistry-grade anti-human Nkx2-3 mAb[31,45] may in the future enable a conclusive assessment to be achieved, even though its expression is probably shared by several mesenchymal cells in a regionally restricted manner of the colonic subepithelial connective tissue (Figure 1).

Involvement of Nkx2-3 in Hematopoietic Malignancies

In addition to exerting important functions in the vascular specification of spleen and Peyer's patches, recently, Nkx2-3 has also been implicated in lymphocyte differentiation. Although lack of Nkx2-3 blocks the development of splenic MZ B cells probably through an altered microenvironment,[35] a recent study showed that the B–cell-restricted overexpression of Nkx2-3 due to the translocation of *NKX2-3* gene to the Ig heavy chain (*IgH*) causes splenic MZ lymphoma. This juxtaposition of 14g32.33 (*IgH*) and 10q24.2 (*NKX2-3*) was identified by break point cloning analysis of B-cell lymphoma samples.[45] In a transgenic (Tg) mouse model using a similar translocation, an oligoclonal expansion occurs first as a result of increased B-cell receptor signaling and enhanced adhesiveness through several adhesion molecules, including LFA-1 integrin, ICAM-1, and MAdCAM-1 adhesion molecules. This condition later evolves into a nongerminal center-type diffuse large B-cell lymphoma (non-GC DLBCL) similar to human mucosa-associated lymphoid tissue and MZ lymphomas. Interestingly, despite the presence of the translocation, the lymphoma usually occurred at a slow pace, beyond 1 year of age in Tg mice. In a large cohort of B-cell malignancies,

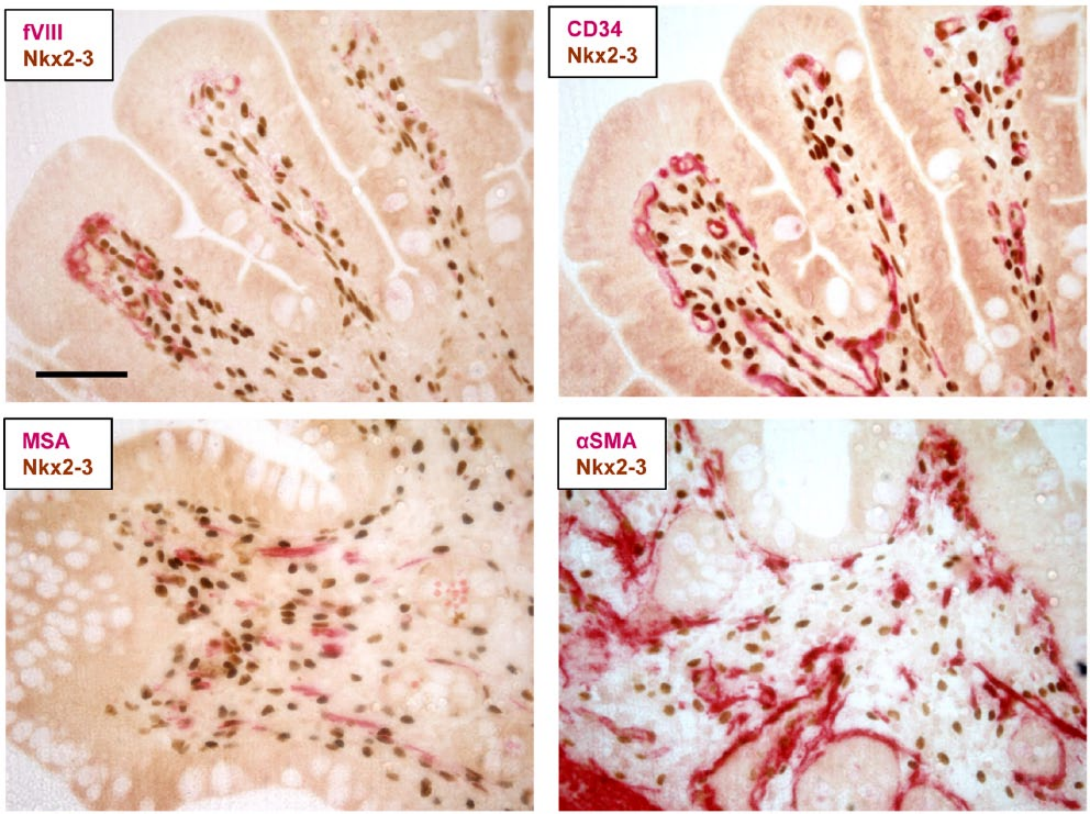

Figure 1. Immunohistochemical localization of Nkx2-3 protein expression in human colon in formaldehyde-fixed paraffin-embedded (FFPE) biopsy. Using reference labeling (in red color) for endothelial markers (factor VIII [upper left] or CD34 [upper right]) or myofibroblast-smooth muscle markers (muscle-specific actin, MSA [lower left] or alpha-smooth muscle actin, αSMA [lower right]) in dual immunohistochemistry reveals both shared expression and partial overlap with Nkx2-3–positive cells (brown nuclear staining). Scale bar, 100 µm.

increased Nkx2-3 expression was detected in 3% of the samples, with higher frequency (6%-7%) in splenic MZ lymphomas and MALT lymphomas, corresponding to the regional expression of Nkx2-3; however, in other forms of B-cell lymphomas without such regional confinement (DLBCL, follicular lymphoma, mantle cell lymphoma, chronic lymphocytic leukemia, or multiple myeloma), upregulated Nkx2-3 was found in less than 1% of the cases.[45] It remains to be seen whether the expression of Nkx2-3 may reflect differences either in the clinical course (including regional involvement) or in therapeutic responses within homogeneous groups of various B-cell malignancies.

Regarding various precursor B-cell alterations induced by Nkx2-3 deviations, the absence of Nkx2-3 in mice caused no marked alteration in the bone marrow B-cell lineage composition.[35] However, in a type of B-cell acute lymphoblastic leukemia (B-ALL) characterized by ETV6/RUNX1 positivity, Nkx2-3 may also be involved. This fusion is created through a t(12;21)(p13;q22) translocation of ETS variant 6 and Run-related transcription factor-1 (RUNX1). A recent study investigated ETV6/RUNX1-positive B-cell precursor-ALL (BCP-ALL)–associated long-coding (lnc) messenger RNA (mRNA) expression patterns, and they identified 16 lnc mRNAs associated with ETV6/RUNX1 in BCP-ALL samples. They also described binding of a histone modification H3K27ac—a regulatory enhancer element—with the ETV6/RUNX1-specific lnc mRNAs such as lnc-NKX2-3-1 in REH cells. Short hairpin RNA silencing of the fusion protein has also confirmed that lnc-NKX2-3-1 and other 3 lnc mRNAs are regulated by ETV6/RUNX1. Although most of these lnc mRNAs have an effect on the translational level, lnc-NKX2-3 and lnc-RTN4R-1 rather act transcriptionally and cause significant alterations of gene expression.[46–48]

In addition to BCP malignancies, Nkx2-3 among other members of NK-like (NKL) homeobox proteins has recently also been involved in T-cell ALL (T-ALL). In cases with blocked *T-cell receptor α-chain* rearrangement, the maturation arrest in a subset of T-ALL was associated with ectopic expression of Nkx2-3 or other members of NKL factors, causing rearrangement inhibition through the repression of *TcRα* enhancer (Eα).[49]

The potential involvement of other Nkx members in T-ALL was previously established, which also demonstrated a connection between TAL1, miR-17-92, and leukemic transformation. *Nkx3-1* may, in a tissue-specific manner, act as tumor suppressor in prostate epithelium[50] but its promoter is a target for TAL1-mediated activation and consequent transformation in T-cell precursors. As downstream target, miR-17-92 is also involved in cell cycle regulation through E2F1 and Notch1-induced T-ALL, although the exact relationship

Table 1. Effects of Nkx mutations on lymphoid organ formation and hematopoietic malignancies.

NKX FAMILY MEMBER	SPLEEN	PEYER'S PATCHES	ASSOCIATION WITH HEMATOPOIETIC MALIGNANCIES
Nkx2.1 (TFF-1)	—	—	Rearrangement in T-cell acute lymphoblastic leukemia[54]
Nkx2.2	—	—	Rearrangement in T-cell acute lymphoblastic leukemia[54]
Nkx2.3	Smaller, atrophic red pulp, disorganized stroma, lymph node like vasculature, defect in B-cell maturation[28]	Fewer, smaller, with altered vascular MAdCAM-1/PNAd switch, abnormal lymphocyte homing[28]	Ectopic expression maturation arrest in T-ALL[49] Involvement in ETV6/RUNX1-positive B-ALL[46] IgH-related translocation in MZBL[45]
Nkx2.5	Asplenia[55]	No contribution[25]	Translocation to BCL11B in T-ALL[52]
Nkx3.1 (BAPX2)	—	—	Induces proliferation in TAL1-positive human T-ALL[50] Ectopic expression in T-ALL cell lines[53]
Nkx3.2 (BAPX1)	Asplenia/hyposplenia[22] Perturbation of LR asymmetry[21]	—	Ectopic expression in T-ALL cell lines[53]

Abbreviations: B-ALL, B-cell acute lymphoblastic leukemia; IgH, Ig heavy chain; LR, left-right; MZBL, marginal zone B-cell lymphoma; T-ALL, T-cell acute lymphoblastic leukemia.

between these participants is not fully determined.[51] In addition, human pediatric T-ALL has also been associated with *Nkx2-5* translocated to either *BCL11B* or *TcRδ*,[52,53] indicating that although in normal T-cell maturation these NK genes play no demonstrable role, they possess transformation potential on ectopic expression.

Conclusions and Perspectives

According to available data, *Nkx2-3* and its paralogues exert essential functions in normal developmental patterning and differentiation of several peripheral lymphoid organs and may play role(s) in hematopoietic/lymphoid malignancies. Although the normal physiological tissue maturation proceeds in a spatially and temporally defined order under the influence of Nkx family members, the involvement of ectopic Nkx2-3 expression in malignant transformation appears to be a random event, coupled with diverse cellular signaling alterations. The most widely established consequence of the deregulated expression of Nkx2-3 is closely linked to IBD, and as this condition represents a possible precursor for colonic cancer, monitoring the tissue expression of Nkx2-3 protein may entail further diagnostic importance (Table 1).

Author Contributions

The review's concept was conceived and the manuscript was written by PB, DV and ZK; immunohistochemistry for Nkx2-3 was contributed by GR and BK; human tissue biopsy samples were provided by ÁV.

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