# Role of Nkx2-3 transcription factor in the vascular-stromal organization of the spleen and intestine

**Doctoral (PhD) thesis**

Dr. Fanni Gábris
Doctoral School of Theoretical Medicine
Mentor: Prof. Dr. Péter Balogh
Head of PhD Program: Prof. Dr. Tímea Berki
Doctoral School Leader: Prof. Dr. Dóra Reglődi

University of Pécs
Clinical Center
Department of Immunology and Biotechnology


Pécs, 2023.

# 1. Introduction

## 1.1 Stromal architecture of the spleen and its role in extramedullary haematopoiesis

The spleen is the largest single lymphoid organ enclosed by a fibrous capsule, structurally divided into two large parts, the white pulp (WP) and the red pulp (RP) (Steiniger 2015). The basic structure of the spleen shows a high degree of similarity between different vertebrate classes and, within mammals, between mice and humans, but there are also many differences between even these two species (Balogh and Lábadi 2010).

Similarly to other peripheral lymphoid tissues, the spleen is also made up of stromal and parenchymal cells. Lymphoid stromal cells are collectively referred to as reticular fibroblasts (FRCs), which are divided into four main groups based on their location and function: marginal zone reticular cells (MRCs), B-cell zone fibroblasts (BRCs), T-cell zone fibroblasts (TRCs) and perivascular reticular cells (PRCs) (Onder, Cheng and Ludewig 2022).

The WP, which is home to T and B cells, plays a major role in adaptive immune response. The RP is involved in the filtering of pathogens and senescent red blood cells and in extramedullary haematopoiesis (EMH). In the resting human spleen, the presence of haematopoietic stem cells (HSCs) can be detected in the red pulp in the immediate vicinity of the sinusoids (Dor et al. 2006, Inra et al. 2015). The local microenvironment created by RP stromal cells is crucial for the maintenance of HSCs (Oda et al. 2018).

## 1.2 The stromal structure of intestinal and colorectal carcinoma and intestinal stem cells

The mammalian gastrointestinal system is of particular importance in nutrition and digestion, absorption and secretion, pH-balance and immunity. The epithelial cells responsible for maintaining the integrity of the intestinal wall are located on the luminal side and are renewed by the division and differentiation of multipotent intestinal stem cells (ISC) in 3-5 days. Underneath the epithelial cells, there is a layer of lamina propria (LP), which is home to many cell types. In this loose connective tissue, different types of fibroblasts, pericytes, blood and lymphatic endothelial cells, neuronal cells and immune cells are present. The lamina propria is separated from the outermost serosal layer of the intestinal wall by the circular and longitudinal smooth muscle layer.

At the bottom of the crypts of the small intestine, about 12-16 ISCs are located between the Paneth cells, which divide to create the transit amplifying zone. The cells found here are characterised by a high proliferative capacity but a short life span. They give rise to progeny differentiated into enterocytes, goblet cells, tuft cells, Paneth cells, and enteroendocrine cells (van der Flier and Clevers 2009). In addition to the conventional ISCs, reserve ISCs are found at position +4 from the base of the crypts, which do not normally behave as stem cells, but can be transformed into leucine-rich repeat-containing G-protein-coupled receptor 5 $(Lgr5)^{+}$ ISCs following severe damage, such as irradiation (Metcalfe et al. 2014). In contrast to the human colon, Paneth cells are not present in the mouse colon (Sasaki et al. 2016, Wang et al. 2020).

The self-renewal and differentiation of ISCs require the cooperation of several microenvironmental signals, transcription factors and metabolic pathways (Zhu, Hu and Xi 2021). Among these, the roles of Wnt/β-catenin, R-spondin, Notch, epidermal growth factor receptor (EGFR) and bone morphogenetic protein (BMP) are the most studied. While the main function of the Wnt signalling pathway is to maintain ISC division, BMP promotes stem cell differentiation. Opposite gradients of Wnt and BMP expression are observed along the crypt axis (Qi et al. 2017).

The characterisation stromal cells of mesenchymal origin located in the intestinal lamina propria is subject of numerous recent researches. Most of the known genes and proteins

described so far are not specific to any single group of stromal cells, and therefore there is often overlap in phenotypic characterisation. Based on scRNA sequencing data in mouse small intestinal stromal cells, mesenchymal stromal cells are characterized by platelet-derived growth factor receptor (PDGFR)α expression, which distinguishes them from αSAM (also known as $Acta2)^{+}$/myosin heavy chain 11 $(Myh11)^{+}$ myofibroblasts (McCarthy et al. 2020, Hsia et al. 2016). In stromal cells, a decreasing gradient of expression PDGFRα from villus towards crypt was described. Based on this, we distinguish $PDGFR\alpha^{high}$ and $PDGFR\alpha^{low}$ groups.

The physiological maturation of ISCs is often perturbed, leading to various cancers. The most commonly diagnosed malignancy of the gastrointestinal system is colorectal carcinoma (CRC), which is the third most common cause of tumour mortality worldwide (Arnold et al. 2017). The tumour microenvironment includes extracellular matrix components, immune cells and endothelial cells, while a significant proportion is composed of tumour-associated fibroblasts (CAF). CAF is an umbrella term that refers to fibroblasts located around invasive tumour and metastasis, regardless of cell origin (Sahai et al. 2020). Their tissue identification is difficult due to the lack of a universal marker to label all CAF cells, the most commonly used markers being fibroblast activation protein (FAP), PDGFR-α, PDGFR-β and Thy-1/CD90. In terms of their origin, they may arise from local fibroblasts, bone marrow-derived mesenchymal cells and other cells of non-stromal origin (such as epithelial, endothelial, pericyte, adipocyte, smooth muscle cell (Sahai et al. 2020, Kalluri 2016)). CAF cells influence tumour progression and regression in different ways. A positive correlation has been shown between the amount of stromal cells surrounding invasive CRC and the mortality rate due to the lesion (Huijbers et al. 2013).

### 1.3 The NK2 transcription class

Tightly regulated gene expression leading to properly established function are essential for the formation of multicellular complex organisms, in which various members of the homeobox (Hox) gene family play a major role. The two largest groups of homeobox genes are the HOX and NK genes (Vojkovics et al. 2018). In contrast to HOX genes, NK genes are not clustered but scattered throughout the genome (Pabst et al. 1997). In Drosophila, 2 classes of NK genes are distinguished. The NK-1 class contains the NK-1 family, and the NK-2 class contains the NK-2, NK-3 and NK-4 families (Vojkovics et al. 2018). Members of the NK-2 family are involved in the development of many organs and tissues. Through the regulation of signalling pathways, they affect cell differentiation, migration and maturation, influencing the development of normal organ structure and the maintenance of organ function (Hombría and Lovegrove 2003). Taken together, mammalian Nkx (human NKX) genes form an extensive network with a tissue-specific expression pattern. Their impaired function leads to dysfunction of many organs and organ systems.

### 1.4 Role of Nkx2-3 transcription factor in the spleen development

Spleen formation in mice is initiated by the establishment of the splanchnic mesenchymal anlagen on embryonic day 10-10.5 (Hecksher-Sørensen et al. 2004). A number of transcription factors are involved in the regulation of this process, such as pre-B cell leukemia transcription factor 1 (Pbx1), Tlx1 (formerly known as HOX11), Nkx2-5 and Nkx3-2 (formerly known as Bapx1 (Brendolan et al. 2005; Brendolan et al. 2007)); in their absence, asplenia develops.

Oliver Pabst and his colleagues observed that the spleens of Nkx2-3 KO mice were smaller than in wild-type mice, with a proportion (about 20%) showing asplenia. In adult mutant mice, the white pulp is reduced in size and no clear follicular structure can be identified. The mutation results in impaired formation of both red pulp and marginal zone sinusoidal endothelial cells (Balogh et al. 2007), and MAdCAM-1 expression which is regulated by the Nkx2-3, is absent

in MZ sinusoidal endothelial cells (Wang et al. 2000, Pabst et al. 2000). In the absence of the lymphotoxin-β-receptor (LTβR)/nuclear factor-κB (NF-κB) signaling pathway or its components (RelB, p52), the formation of marginal zone sinusoidal endothelial cells is also inhibited; however, the development of red pulp sinusoidal endothelial cells is unimpaired (Balogh et al. 2007).

In the spleens of Nkx2-3$^{-/-}$ mice, high-endothelial venule (HEV)-like vascular structures appear, formed in an LTβR-dependent process (Czömpöly et al. 2011). These vascular structures undergo postnatal maturation, during which the MAdCAM-1 addressin is gradually replaced by the appearance of peripheral lymph node addressin (PNAd). PNAd$^{+}$ endothelial cells produce CCL21 chemokine and mediate the homing for L-selectin ligand-containing lymphocytes. A lymph node-like shift in the T/B cell ratio was also observed in the mutant spleen (Czömpöly et al. 2011).

In the absence of Nkx2-3, lymphocyte-filled lymphatic endothelial hyaluronan receptor 1 (LYVE-1) positive vascular sacs appear in the spleen (Kellermayer et al. 2011). qPCR analysis of these spleens showed a significant increase in LYVE-1 and podoplanin/gp38 mRNA levels without an increase in vascular endothelial growth factor receptor type 3 (VEGFR-3) and prospero homeobox protein 1 (Prox-1) mRNA levels, the latter two being specific for lymphatic endothelial cells (LECs).

In the absence of Nkx2-3, the structural abnormalities observed in the spleen are associated with a decrease in the immunological functions. The absence of MAdCAM-1 addressin in marginal sinus endothelial cells is coupled with a reduced lymphocyte recirculation and impaired macrophage presence, leading to impaired pathogen clearance (Balogh et al. 2007, Czömpöly et al. 2011). Furthermore, in the absence of Nkx2-3, a maturation defect in B cells is observed, resulting in impaired T-dependent antibody response and affinity maturation (Tarlinton et al. 2003).

### 1.5 The role of Nkx2-3 in gut development

In 1997 Oliver Pabst and colleagues reported that Nkx2-3 mRNA is present in the mesoderm of the midgut and hindgut in mouse embryos, and its expression is detectable in the postnatal period (Pabst et al. 1997). It has been observed that more than half of Nkx2-3 homozygous mutant mice die at weaning period, which has been suggested to be due to impaired development of the small intestine (Pabst et al. 1999). In embryonic histological sections, the number of villus in the jejunum and ileum is reduced, accompanied by a thinned mesenchymal layer. These lesions persist in the early postnatal period but are not observed in the duodenum and colon.

The length of the small intestine and colon of adult Nkx2-3 mutant mice was maintained, but the diameter of the intestinal segments was increased and vascularization was enhanced in the absence of the gene. In the small intestine, elongated, thin villuses and extensive crypts are present, with increased cell division and normal levels of apoptosis compared to controls. These results raise the possibility that mesenchymal Nkx2-3 expression affects epithelial cell division (Pabst et al. 1999).

In the absence of Nkx2-3, fewer and smaller Peyer's patches are present in the small intestine. Postnatally, MAdCAM-1 is expressed on the HEV surface of Peyer's patches and is involved in the mucosal homing process of lymphocytes via the α4β7 integrin. In the absence of Nkx2-3, MAdCAM-1 gradually disappears from the endothelial cell surface and is replaced by PNAd, which may explain the reduced Peyer's patches size in the absence of Nkx2-3 (Wang et al. 2000, Kellermayer et al. 2014). Deletion of Nkx2-3 also affects the intestinal distribution of ILC type 3 (ILC3) cells.

## 2. Objective

In my research, I aimed to investigate the role of the Nkx2-3 protein in splenic red pulp organisation and its role in extramedullary haematopoiesis. I was interested to explore the origin of the LYVE-1 positive vascular formations. Furthermore, I investigated the expression of NKX2-3 protein in different ages intact, pre-malignant and malignant human colon samples. Experiments were performed to phenotype NKX2-$3^{+}$ stromal cells.

We aimed to answer the following questions:

**A) Effect of Nkx2-3 deficiency on spleen endothelial cells and red pulp:**

**A1, What is the origin of LYVE-1 positive sac-like formations in the spleen in the absence of Nkx2-3?**

**A2, In addition to the known structural abnormalities, what are the effects of Nkx2-3 deficiency on splenic red pulp sinus endothelial cells?**

**A3, What is the effect of Nkx2-3 deficiency on extramedullary red blood cell formation?**

**A4, Does the absence of Nkx2-3 affect the incidence and expansion of megakaryocytes in the spleen?**

**B) Role of NKX2-3 in the organization of large intestinal stromal elements:**

**B1, Are there any differences in the expression of the NKX2-3 transcription factor in intact human colon histology samples from different age groups?**

**B2, Which stromal element(s) can be associated with NKX2-3 protein expression in human colon?**

**B3, Is there any alteration of NKX2-3 expression in pre-malignant and malignant human colon processes?**

# 3. Materials and methods

## 3.1 Experimental animals

Young adult (8-10 weeks of age) Nkx2-3$^{-/-}$ and wild-type BALB/c female mice were used to study stress haematopoiesis. To induce stress haematopoiesis, blood was collected by puncture of the lateral tail vein three times with 2-day recovery periods. On the seventh day, mice were terminated, their blood was collected for cellular and serological analyses, while bone marrow and spleen were processed for flow cytometry and immunohistochemical analysis. The megakaryocytes were stimulated with Romiplostim (Sparger et al. 2018, Slayton et al. 2002). Spleens were processed on the seventh day after treatment. Prox1$^{GFP}$-Nkx2-3$^{-/-}$ animals were used to identify LECs (Choi et al. 2011).

Mouse experiments were carried out in the animal facility of the Department of Immunology and Biotechnology, University of Pécs, under license numbers BA02/2000-16/2015 and BA02/2000-43/2021, with approval for the use of genetically modified organisms under license number SF/27-1/2014 issued by the Ministry of Rural Development, Hungary, in accordance with the guidelines set out by the Ethics Committee on Animal Experimentation of the University of Pécs, Hungary.

## 3.2 Flow cytometry

The cellular composition of bone marrow and spleen was determined by flow cytometry. Bone marrow was extracted from femoral bones. Spleen cells were extracted using 2 slides and filtered on cotton wool. Cell counting was performed using a LUNA-II automated counting machine. The cell suspension was incubated with different labelled and unlabelled rat monoclonal antibodies on ice for 20 min. Unlabeled antibodies were detected with labeled anti-rat secondary antibodies. Samples were fixed with PBS solution containing 1% formaldehyde.

For the analysis of LECs, lymph node and spleen samples from Prox1$^{GFP}$-Nkx2-3$^{-/-}$ mice were digested with DNAse I/Liberase (Roche) cocktail, after labeling with different monoclonal antibodies, dead cells were excluded using 7-aminoactinomycin D (7AAD).

Samples were measured with a BD FACSCalibur flow cytometer and analysed with FCS Express v.6 and FlowJo w10.

## 3.3 Immunofluorescence and immunohistochemistry in animal experiments

8 μm thick sections from snap-frozen mouse spleens were prepared and fixed with cold acetone after drying. For immunofluorescence labeling, our samples were incubated with labeled and unlabeled antibodies at room temperature for 45 min. For immunohistochemistry, endogenous peroxidase inhibition was performed with 0.1% phenyl-hydrazine solution. Our sections were labeled with different antibodies. Our samples were incubated with ImmPRESS goat anti-rat IgG peroxidase conjugate, and the reaction was visualized with DAB-$H_2O_2$ substrate mixture. For nuclear staining, Mayer's hematoxylin was used and after dehydration, the staining was covered with Pertex medium.

Pictures of the histological samples were taken using an Olympus BX61 microscope and ZEN software. Post-processing of the images was done with Photoshop. For statistical analysis, our immunohistochemical samples were digitized using a Pannoramic View scanner (3D Histech, Budapest) and evaluated using QuPath-0.2.3 software.

Whole spleen samples from Prox1$^{GFP}$-Nkx2-3$^{-/-}$ and control heterozygous mice were fixed in 4% paraformaldehyde solution and then CUBIC method (Susaki et al. 2014) was used to make them transparent. Spleen preparations were examined using an Olympus FluoView FV1000 laser scanning confocal microscope (Olympus Europa SE & Co., Hamburg, Germany).

3D images were acquired using Z-stack data using Imaris Software (Bitplane AG, Zurich, Switzerland).

### 3.4 Blood parameters and EPO ELISA

For the analysis of blood parameters, 150-200 µl blood was collected in 0.25/0.5 ml MiniCollect Complete blood collection tubes containing K3 EDTA. Blood samples were measured and analyzed using a Sysmex XN-V1000 hematology automat and its software package. Serum erythropoietin levels were determined by Legend Max mouse EPO ELISA kit (BioLegend, Biomedica Hungaria, Budapest).

### 3.5 Criteria for sample selection

Sample materials were selected from the diagnostic tissue archive of the Department of Pathology, Clinical Center of the University of Pécs. For the studies, we established five categories, including (A) healthy neonates and infants (0-3 years, average age 1.3 years, n=10); (B) healthy adolescents (15-20 years, average age 17.9 years, n=5); (C) healthy adults and seniors (50-80 years, average age 65.8 years, n= 10); (D) patients with colonic polyps (46-73 years, average age 62.5 years, n=10) and (E) patients with CRC (63-88 years, average age 71.7 years, n=15). Tissue samples were considered healthy if they were devoid of malignant, hypoxic/necrotic or inflammatory lesions, examined by experienced pathologists. The histopathological diagnosis of polyp samples was adenomatous polyp with low-grade dysplasia, while all tumors were adenocarcinomas. The ethical permit under license number 8578-PTE 2020 was issued by the Regional Research Ethical Committee of the University of Pécs Clinical Center.

### 3.6 Histological analysis of human samples

Sections were scanned using a Pannoramic Midi scanner (3D Histech Ltd, Budapest, Hungary) and were then analyzed using QuPath-v0.2.3. software. In the lamina propria layer of sections, we randomly chose five different representative regions containing approximately 150-200 nucleated cells, counted automatically by the software. Cells were assigned into four groups based on their nuclear DAB staining optical density (OD): negative: DAB OD mean <0.2; weakly positive: DAB OD mean 0.2-0.4; moderately positive: DAB OD mean 0.4-0.6; strongly positive: DAB OD mean > 0.6. During analysis, all nuclei were counted and grouped according to their labeling intensities. For the analysis of dual immunohistochemistry samples, the red, brown and blue reaction products were digitally separated with color deconvolution of the RGB images, followed by the manual counting of cells. Samples were also evaluated according to their histoscore (H-score) feature, calculated as H-score = 1 x % weak + 2 x % moderate + 3 x % strong nuclei, with values ranging between 0-300.

### 3.7 2.5. Statistical analysis

The statistical analysis of samples was performed using GraphPad Prism9. Statistical significance was determined using Mann-Whitney U-tests or Fisher's exact test. ns, $p>0.05$; *, <0.05; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

# 4. Results

## 4.1 Lack of Nkx2-3 leads to the appearance of ectopic lymphatic vessels in the spleen

In the absence of Nkx2-3 in the spleen, LYVE-1 positive vascular bundles can be identified, surrounded by a fibroblast network labelled with ER-TR7 mAb (anti-mouse collagen VI, (Schiavinato et al. 2021)). However, neither immunohistochemistry nor mRNA expression studies have been able to detect increased Prox-1 expression to support a lymphatic endothelial origin (Kellermayer et al. 2011). Prox-1 is a major regulatory protein during LEC-directed differentiation, while LYVE-1 is present on serosal macrophages and the surface of splenic megakaryocytes in addition to lymphatic endothelial cells. Therefore, we aimed to determine the tissue origin of LYVE-1-positive sacs by investigating eGFP expression driven by Prox-1

### 4.1.1 Appearance of ectopic *bona fide* lymphatic vessels in Nkx2-3$^{-/-}$ spleen

Previously we found that, in addition to the lymph node-like shift of addressin preference from MAdCAM-1 in marginal sinus-lining cells to PNAd displayed by ectopic high endothelial venules (HEVs) (Czömpöly et al. 2011), the spleens of Nkx2-3-deficient mice also contain lymphocyte-filled vascular sacs expressing LYVE-1 LEC-associated hyaluronan receptor, surrounded by a fibroblastic meshwork identifiable by ER-TR7 mAb against Collagen type VI (Kellermayer et al. 2011;Schiavinato et al. 2021). As LYVE-1 can also be expressed by non-LECs, such as serosal macrophages or splenic megakaryocytes (Schledzewski et al. 2006), and our previous anti-Prox1 immunohistochemical results provided conflicting results, next we also tested Prox1 expression in Nkx2-3-mutant spleens as master regulator for LEC specification by backcrossing Nkx2-3$^{-/-}$ mice with *Prox1*$^{GFP}$ mice. First, we investigated whole-mount spleen samples from Nkx2-3 deficient and heterozygote mutant mice, using the CUBIC optical clearing procedure (Susaki et al. 2014). We found that this treatment effectively rendered the *Prox1*$^{GFP}$-Nkx2-3$^{-/-}$ spleens completely transparent, while in heterozygote and wild-type samples some residual brownish hemoglobin-derived pigment remained. In *Prox1*$^{GFP}$-Nkx2-3$^{-/-}$ mice, we found an extensive arborized meshwork of Prox1-positive tubes, with their diameter ranging between 15-40 μm. These structures often created anastomoses between branchings, establishing ring-like connections, but revealed no valve-like formations, indicating initial lymphatic capillaries (Lutter et al. 2012). In contrast, in Nkx2-3 heterozygote spleens with Prox1 reporter expression we only occasionally observed low-level GFP$^+$ signal associated either to macrophages or due to background autofluorescence, without any evidence for capillary formation. Next, we used flow cytometry to confirm that, in *Prox1*$^{GFP}$-Nkx2-3$^{-/-}$ mice, the splenic GFP$^+$ cells are identifiable as LECs. Indeed, GFP$^+$ cells were CD45$^-$gp38$^+$CD31$^+$, corresponding to LECs normally present in lymph nodes (Link et al. 2007). In contrast, the GFP$^-$CD45$^-$ cells (also excluding the 7AAD-positive dead cells) included gp38$^-$CD31$^-$, and gp38 or CD31 single positive mesenchymal cells, corresponding to the follicular or other stromal cells including follicular dendritic cell (FDCs) and pericytes, T-zone reticular fibroblasts (FRCs) or blood endothelial cells (BECs) as main stromal subsets, respectively. With these flow cytometric settings, we were unable to identify any GFP$^+$CD45$^-$ live cells in spleens of Nkx2-3 heterozygous mice. These finding indicate that in *Prox1*-GFP$^+$ Nkx2-3-deficient spleens, gp38$^+$/CD31$^+$ double positive LECs are the only GFP-positive cells. Based on these observations, we conclude that the RP vasculature of the spleen in the absence of Nkx2-3 is transformed to harbor ectopic lymphatic capillary meshwork with gp38/CD31 double positive cell surface phenotype and Prox1-expression corresponding to LECs.

### 4.1.2 Absence of Nkx2-3 causes defective red pulp vascular specification of the spleen

The presence of lymphatic capillaries in the spleen of mice lacking Nkx2-3 suggests a major shift in the splenic vasculature, with such structures typically lacking in wild-type mice. We have previously observed a significant reduction of red pulp (RP) venous sinus vessels identifiable with IBL-9/2 mAb (Balogh et al. 2007). To further examine how vascular alterations in this genotype affect other specific traits of this regional vasculature, next we investigated the expression of Clever1 scavenger receptor encoded by Stab1 gene. This endocytic receptor on LECs in wild-type mice is also expressed by non-continuous BECs involved in splenic B-cell homing to RP (Prevo et al. 2004;Tadayon et al. 2019). Using immunofluorescence microscopy, we found that in wild-type mice, Clever1 expression in the RP vasculature largely overlaps with the reactivity of IBL-9/2, whereas the absence of Nkx2-3 abolished the sinusoidal labeling for both markers. In contrast, although Stab1$^{-/-}$ mice lack Clever1, the RP sinus reactivity of IBL-9/2 mAb remained unaltered. These findings indicate that the vascular shift elicited by the absence of Nkx2-3 affects both the maturation of RP sinus meshwork containing BECs into Clever1$^{+}$ segments, and the differentiation of ectopic LEC capillaries.

### 4.1.3 Disrupted vascular stroma in Nkx2-3-deficient spleen impairs extramedullary stress hematopoiesis

In mice the RP vasculature plays an important role in extramedullary hematopoiesis as RP sinusoidal endothelial cells, in association with Tcf21$^{+}$ stromal cells, create a niche for hematopoietic stem cells by providing them with stem cell factor/KitL and CXCL12 (Inra et al. 2015). As in Nkx2-3$^{-/-}$ spleens we observed a defective RP vasculature and ectopic lymphatics, we were interested in whether this shift has any effect on the hematopoiesis-supporting stroma. Although the various stromal components in the splenic RP are not as clearly delineated phenotypically as in the white pulp (Lim and O'Neill, 2019;Bellomo et al. 2020), the extramedullary hematopoietic activity of the splenic RP has been suggested to involve CD29-positive stromal cells (O'Neill et al. 2019). Moreover, the RP reticular stroma as well as endothelial cells also broadly display VCAM-1. Therefore, next we investigated the presence of stromal cells by multicolor immunofluorescence of these stroma markers. We found that both CD29 and VCAM-1 in normal BALB/c mice were expressed dominantly by RP stromal constituents, in addition to blood vessels. The CD29$^{+}$/VCAM-1$^{+}$ extravascular stromal cells form a meshwork throughout the RP (Lim and O'Neill, 2019;O'Neill et al. 2019). In Nkx2-3 mutants, we could also detect CD45-positive regions intermingled with both vascular and non-vascular CD29$^{+}$/VCAM-1$^{+}$ cells. However, in Nkx2-3-deficient mice, we observed that although both stromal and vascular CD29$^{+}$/VCAM-1$^{+}$ cells are present, the RP stromal compartment is fragmented, without a definable red pulp-white pulp boundary.

Next, we investigated whether the vascular-stromal alterations observed in the RP affect the reserve hematopoietic potential of the spleen. First, we measured basic blood parameters to obtain a general overview of the hematopoietic function of mice lacking Nkx2-3. These parameters revealed chronic anemia in Nkx2-3 deficient mice, manifested as significantly lower red blood cell (RBC, *p=0.008*), hematocrit (HCT, *p=0.0143*) and hemoglobin (HGB, *p=0.0073*) levels in Nkx2-3$^{-/-}$ mice compared to wild type BALB/c mice. In contrast, the absolute number of total white blood cells, including both neutrophils (*p=0.0007*) and lymphocytes (*p=0.008*) was significantly increased. Blood loss induced by repeated blood withdrawals led to significantly lower RBC (*p=0.02*) and HGB (*p=0.0135*) levels in BALB/c mice, while the hematocrit was unchanged. In Nkx2-3$^{-/-}$ mice, we observed a similar pattern of moderately reduced hematocrit without reaching statistically significant difference, but with significantly lower RBC (*p=0.0303*) and HGB (*p=0.0173*) levels. Importantly, in treated Nkx2-

$3^{-/-}$ mice all three values were significantly lower, compared to treated wild-type mice. To rule out that in Nkx2-3-deficient mice decreased EPO production is responsible for reduced erythropoiesis, we compared EPO levels in untreated and bled BALB/c and Nkx2-3 mutant mice by ELISA. We found that in untreated mice lacking Nkx2-3 the basal EPO level was significantly higher compared to BALB/c mice (*p=0.0011*), and upon blood loss, this further increased, significantly exceeding that of treated BALB/c mice. This finding also indicates that under steady-state conditions, the increased level of EPO is insufficient to maintain a normal hematocrit level, and the significantly augmented EPO production fails to achieve erythropoietic recovery in mice lacking Nkx2-3.

To define the extramedullary erythropoietic capacity in spleen, the frequency and absolute number of splenic TER-$119^{+}$/$CD45^{-}$ erythroid lineage cells were determined by flow cytometry in Nkx2-$3^{-/-}$ and wild-type BALB/c mice, and compared to bone marrow after repeated bleeding. We found that in untreated Nkx2-$3^{-/-}$ mice the frequency and absolute number of splenic erythroid cells were lower compared to BALB/c mice. After bleeding the spleen in Nkx2-3 mutant mice failed to reactivate its erythropoietic activity, while in BALB/c mice a robust erythroid expansion occurred. In the bone marrow, the total cell number was lower in Nkx2-$3^{-/-}$ mice compared to BALB/c mice, and following bleeding it did not change significantly in BALB/c mice, whereas in Nkx2-$3^{-/-}$ mice the total cell number significantly (*p=0.0189*) decreased. In bone marrow the ratio of TER-119-positive erythroid cells was significantly higher in untreated Nkx2-$3^{-/-}$ mice than in wild-type controls (*p=0.0079*), and their frequency significantly increased in both genotypes following bleeding. In Nkx2-3-deficient mice this increase of TER-119-positive cell percentage was significantly higher; however, the increase in absolute numbers was significant (*p=0.0046*) only in BALB/c mice.

Immunohistochemical analyses in untreated BALB/c mice revealed TER-119-positive erythroid clusters within the red pulp-restricted CD29-positive stromal meshwork, in addition to the erythrocytes trapped in the RP; however, such clusters were absent in Nkx2-3-deficient spleen, only erythrocyte clumps were present. Following blood withdrawal, in BALB/c mice the TER-119-positive erythroid colonies become confluent, virtually completely filling the tissue space between the neighboring white pulp regions. In contrast, in Nkx2-3-deficient mice following the loss of blood the expansion of RP TER-119-positive colonies was completely abolished, although CD29-positive extravascular reticular cells were present, indicating the tissue-specific functional impairment of erythropoietic support. These results establish that, in addition to the reduced steady-state splenic erythropoiesis, the induced stress hematopoiesis is also significantly impaired in Nkx2-3 deficient mice in a tissue-specific manner.

### 4.1.4 Absence of Nkx2-3 blocks the splenic megakaryocyte expansion following TPO-agonist treatment

In addition to producing erythroid cells, the splenic RP in mice also harbors megakaryocytes, as a RP-dwelling non-lymphoid resident hematopoietic lineage (Noetzli et al. 2019). To test if the defective extramedullary hematopoiesis also affects the occurrence of splenic megakaryocytes and their expansion, we next investigated the effect of intravenously injected Romiplostim, a peptide-fusion protein acting as TPO receptor agonist stimulating megakaryocytes (Léon et al. 2012). The effect was determined by the morphometric quantification of the splenic megakaryocyte numbers, following immunohistochemical staining for CD41.

In untreated BALB/c mice, megakaryocytes in the RP localized frequently in the subcapsular regions as single cells or doublets. In contrast, in Nkx2-3-deficient mice only occasional megakaryocytes were present, also typically in subcapsular locations. Romiplostim treatment led to a robust increase of platelet-associated labeling by anti-CD41 staining in wild-type

spleens significantly blurring the identification of megakaryocytes. We observed only minimal increase in Nkx2-3-deficient spleens. Quantification of megakaryocytes by Pannoramic Viewer scanner and QuPath-0.2.3 software based on their nuclear morphology revealed an approximately tenfold increase for megakaryocyte number in BALB/c spleen (*p=0.0053*). In contrast, such an increase was absent from Nkx2-3-deficient mice. Together these data indicate that while the residual RP in Nkx2-3-deficient mice may harbor a reduced number of megakaryocytes, the spleen cannot expand its megakaryocyte population following TPO-receptor agonist stimulation, in contrast to wild-type BALB/c mice.

### 4.2 NKX2-3 expression in human colorectal tissue samples

In addition to the spleen, the primary site of NKX2-3 expression is the intestinal tract, where a number of functional data suggest a role for NKX2-3 factor in local lymphoid tissue development and in ensuring lymphoid tissue homeostasis. In contrast, the expression of NKX2-3 in human tissues is currently poorly understood, and therefore we investigated the tissue characteristics of NKX2-3 production in human intestinal samples.

#### 4.2.1 Distribution and ratio of NKX2-3$^+$ cells in healthy human colon

First, we investigated the expression of NKX2-3 by analyzing normal human colon tissues with immunohistochemistry. We considered the biopsy samples normal if no inflammatory, necrotic or neoplastic features were present. The majority of cells with nuclear NKX2-3 staining were located in the tunica mucosa and, to a lesser degree, also in the tunica muscularis, while the nuclei of epithelial cells were consistently negative for NKX2-3.

As pericryptal NKX2-3$^+$ cells have been shown to influence the intestinal stem cell niche (Hsia et al. 2016), in our subsequent studies we focused on analyzing the NKX2-3-positive cells within the lamina propria. Here we found that the NKX2-3$^+$ nuclei were homogenously dispersed along the crypts. Next, we quantified the presence and the distribution of NKX2-3-positive cells at various ages. We found that within the lamina propria, the frequency of NKX2-3-positive nuclei was highest in the 0-3 years age group, and was significantly lower in both the 15-20 years age group and 50-80 years age group (*p=0.0127* and *p=0.0039*, respectively). The frequency of NKX2-3-positive cells did not differ significantly between the 15-20 years and 50-80 years age groups.

The immunohistological stainings revealed various nuclear NKX2-3 labeling intensities. Therefore, we quantified the percentage of NKX2-3-positive cells with weak, moderate, or strong expression, and compared the frequency (defined as percentage of counted NKX2-3$^+$ lamina propria nuclei) of various labeling intensities between different age groups. In general, we observed an overall dominance of strongly positive nuclei in each age group, although the higher percentage of strong NKX2-3-expressing cells reached statistical significance only among the 15-20 years age group (*p=0.0079* compared to moderate and *p=0.0079* compared to weak intensities, respectively).

As a measure of overall NKX2-3 presence, we calculated the H-score which combines the frequency of NKX2-3$^+$ cells with intensity of nuclear NKX2-3 expression. We observed a significantly higher H-score in the 0-3 age group compared to the 50-80 years group (*p=0.0185*). Collectively, these findings confirm our previous results on the reproducible immunohistochemical detectability of NKX2-3 protein in human biopsy samples (Vojkovics et al. 2018) in the tunica mucosa and tunica muscularis layers, also revealing the dominance of strong labeling in all age groups, with the highest frequency and H-score in the 0-3 years age group.

### 4.2.2 Characterization of NKX2-3$^+$ cells.

Due to the dispersed pattern of labeling described above, we assumed that several cell types express NKX2-3. Based on our previous dual labeling data for Factor VIII, CD34, muscle-specific actin (MSA) and α-smooth muscle actin (αSMA) (Vojkovics et al. 2018), we hypothesized that both endothelial and a substantial fraction of non-endothelial mesenchymal cells may share NKX2-3 production. In mice, a gp38$^+$ pericryptal mesenchymal stromal subset also expresses CD34, in addition to endothelial cells (Stzepourginski et al. 2017). For a more detailed characterization of NKX2-3-positive mesenchymal cells in human colonic samples, we investigated the presence of NKX2-3 in subepithelial myofibroblasts that were previously found to express αSMA (Adegboyega et al. 2002). Therefore, we next aimed to define the lineage-affiliation of NKX2-3 expression in the pericryptal rim of the lamina propria. Due to the stronger staining of NKX2-3, we used samples from healthy 0-3 years age group for the lineage analysis.

First, corroborating our previous observations, we found that αSMA is expressed in the colonic pericryptal rim, in addition to its expression in the deeper regions of the lamina propria and the smooth muscle; however, its blurred staining pattern hampered the precise identification of the pericryptal region adjacent to the epithelium.

To study the organization of this region in further details, we used other mesenchymal markers. We found individual CD34$^+$ pericryptal cells co-expressing NKX2-3. Careful analysis indicated that these non-vascular CD34$^+$ cells could be resolved into NKX2-3-positive and NKX2-3-negative subsets, sometimes even represented by two neighboring cells. Morphometric analysis revealed that only 22.4 percent (234 out of 1045) of Nkx2-3-positive pericryptal cells expressed CD34. In a reverse fashion, 75% of CD34-positive cells (234 of 312) in this region displayed NKX2-3, while the remaining 25% of CD34$^+$ cells (78 out of 312) were NKX2-3-negative; in addition, CD45$^+$ leukocytes also lacked NKX2-3 expression.

In mice vascular adhesion protein-1 (VAP-1/AOC3) has been reported to identify Nkx2-3$^+$ pericryptal myofibroblasts (Hsia et al. 2016), while in healthy human colon it is also expressed in the vasculature of lamina propria and submucosa (in addition to the smooth muscle cells of the lamina muscularis mucosae), thus prompting the analysis of its correlation with NKX2-3. In the pericryptal regions, we could observe NKX2-3/VAP-1 double positive cells. Furthermore, in the deeper regions of lamina propria we also found vascular endothelial labeling of VAP-1. In the pericryptal zone, the majority of local VAP-1 positive cells also expressed NKX2-3, and the comparison of the degree of overlap between NKX2-3/VAP-1 to that of NKX2-3/CD34 revealed that a larger fraction of NKX2-3-positive cells displayed VAP-1 (340 out of 669, 50.8%) than those expressing CD34. Statistical comparison indicated that VAP-1 expression is more closely related to the production of NKX2-3 compared to CD34, as only 5.1% of VAP-1 positive cells (21 out of 361) lack NKX2-3 labeling, while as shown above, 25% of CD34$^+$ lacked NKX2-3 ($p<0.0001$).

To identify NKX2-3 positive lamina propria mesenchymal cells lacking all of these endothelial/myofibroblastic markers, we next performed dual labeling with a cocktail of anti-CD34/VAP-1/αSMA antibodies visualized with the same red precipitate, and correlating it with NKX2-3 detection via brown DAB chromogen. We found that although the majority of NKX2-3$^+$ cells also stained positive with the anti-CD34/VAP-1/αSMA cocktail, NKX2-3 single positive cells were still readily detectable. Similarly to the extensive labeling observed for evaluation of αSMA staining our attempts to quantify the overlap with NKX2-3 staining were futile. Taken together, our results reveal that, although the majority of histologically detectable NKX2-3$^+$ cells can be assigned to various pericryptal myofibroblast lineages, none of the markers employed appears to be able to uniquely identify the NKX2-3$^+$ cells; in addition, a small fraction of NKX2-3$^+$ cells do not display CD34, VAP-1 or αSMA.

### 4.2.3 Distribution and ratio of NKX2-3$^+$ cells in colorectal cancer samples.

Previous reports identified polymorphisms in NKX2-3 as a susceptibility trait associated with inflammatory bowel diseases (Parkes et al. 2007), and its absence in mice resulted in enhanced epithelial proliferation in both homeostatic and inflammatory conditions (Kellermayer et al. 2019), thus raising its potential involvement in epithelial proliferative conditions, including polyps and adenocarcinomas. Therefore, next we examined the expression of NKX2-3 in patients with either pre-malignant adenomatous polyps or malignant adenocarcinomas. The cohort of normal 50-80-year old samples was used as age-matched controls.

Similarly to non-tumor samples, NKX2-3$^+$ cells were homogenously distributed throughout the lamina propria of polyp and adenocarcinoma samples. Comparing the frequency of NKX2-3$^+$ nuclei relative to the total number of nuclei, we observed a lower percentage in polyps compared to non-tumor controls ($p=0.0433$), and this frequency further decreased ($p=0.0003$) in adenocarcinoma samples. On the other hand, the difference between polyp and adenocarcinoma samples did not reach statistical significance.

To dissect whether the reduction of NKX2-3 positive nuclei is a general phenomenon or is related to a shift in labeling intensity, we analyzed the distribution of nuclei with various NKX2-3 expression levels. In general, we found that in both polyp and adenocarcinoma samples weakly labeled NKX2-3$^+$ cells represented the largest proportion of nuclei. The percentage of these cells was significantly increased compared to healthy controls, coupled with significant decreases in the moderately and strongly NKX2-3$^+$ nuclei. This was in contrast to the findings in healthy controls where intensely NKX2-3$^+$ cells represented the largest fraction of cells.

In line with the reduced number and labeling intensity of NKX2-3 positive nuclei, we also observed a significantly lower H-score in both polyp and CRC samples compared to those in healthy samples ($p=0.0288$ and $p=<0.0001$, respectively). Moreover, in CRC samples the H-score was significantly lower ($p=0.0096$) compared to polyp samples.

Taken together, these findings establish the loss of cells with strong NKX2-3 expression in patients with adenomatous polyp or with defined colonic adenocarcinoma.

# 5. Discussion

During my PhD work, I investigated the role of the Nkx2-3 transcription factor in the organisation of the splenic red pulp and the origin of the LYVE-1 positive vascular sacs. I analysed NKX2-3 protein expression in intact colon histological sections from different age groups and performed experiments to phenotype NKX2-3+ stromal cells. Based on literature data, NKX2-3+ stromal cells have a role in the formation of the intestinal stem cell microenvironment, therefore our results from intact histological specimens were compared with the results from pre-malignant and malignant lesions.

In this work, we describe the identification of ectopic LEC capillaries displaying lymphatic endothelial cell (LEC) surface markers and Prox1 fate-determining transcription factor in splenic vasculature in mice lacking Nkx2-3, coupled with defective stromal differentiation and support capacity of splenic stress hematopoiesis. These observations confirm Nkx2-3 as a critical factor for both tissue-specific lymphatic-blood endothelial commitment as structural elements, and as essential components to maintain the stress hematopoietic capacity of spleen.

After the separation of splanchnic mesenchyme, the emerging spleen anlage is arranged around its blood vasculature, surrounded by specialized stromal constituents, also accompanied by regional macrophage subsets (Bellomo et al. 2021, Steiniger et al. 2007). Recent analyses of splenic stromal subpopulations using scRNAseq combined with lineage tracing and phenotypic identification have revealed substantial functional differences coupled with complex tissue location preferences, particularly for white pulp fibroblastic reticular cell subsets (Alexandre et al. 2022, Cheng et al. 2019). Typically, these analyses address the reticular scaffolding of the various splenic compartments, although the blood endothelial subsets also display considerable heterogeneity, including phenotypic markers, chemokine profile and adhesion molecules (Balázs et al. 2001, Berahovich et al. 2014, Tadayon et al. 2019). Despite the earlier observations demonstrating the presence of deep efferent lymphatic vessels in mice (Pellas and Weiss 1990, Steiniger and Barth 2000), analyses addressing the origin of putative splenic LECs are scarce. The exact origin of these splenic lymphatic vessels in humans is currently unknown, most likely owing to their much less obvious presence (through the use of lymphatic markers including LYVE-1, podoplanin or Prox1) compared to other lymphoid tissues, such as lymph nodes, Peyer's patches, or dermal and intestinal lymphatics.

Using scRNAseq approach also demonstrated that Nkx2-3 is amongst the most distinctive genes characteristic for the spleen (Alexandre et al. 2022). Its mRNA expression is also shared by various splenic white pulp reticular cell subpopulations - comprised of at least 8 different mesenchymal subsets, in addition to vascular-related adventitial and pericyte as well as mesothelial cells - located within the white pulp and marginal zone. In contrast, the red pulp stromal subsets (while constituting a large part of total splenic stromal cells) were less divergent, and analyzing a public database (accessible at http://muellerlab.mdhs.unimelb.edu.au/frc_scrnaseq/) for their Nkx2-3 expression presents only a moderate expression of Nkx2-3.

Earlier immunohistochemical findings from our laboratory demonstrate that in human spleen samples the expression of Nkx2-3 is restricted to RP endothelial cells (Kellermayer et al. 2016). This distribution mirrors the drastic reduction of RP vessels identifiable by IBL-9/2 mAb and displaying Clever1 (encoded by Stab1) in Nkx2-3 deficient mice. Recent comprehensive expression analyses reveal that Nkx2-3 mRNA is selectively expressed in splenic, small intestinal and colonic vascular endothelium (Kalucka et al. 2020). Interestingly, however, neither Nkx2-3 nor Stab1 is included in the top-50 expressed genes in various vascular segments of the spleen, while Prox1 is listed, but without Lyve1. Our present findings demonstrate evidences that RP blood vessels are largely replaced by ectopic Prox1-positive lymphatic

capillaries, indicating a defining role for Nkx2-3 in vascular patterning (including lymphatic vs. blood endothelium commitment) of the spleen.

What can the explanation be for this eventual shift of RP vasculature into putative lymphatic capillaries? In the HEVs of intestinal lymphoid tissue vasculature, Nkx2-3 activates the promoter of MAdCAM-1 addressin through forming a composite element together with DNA-binding orphan nuclear receptor COUP-TFII, a critical transcription factor defining venous endothelial identity through suppressing Notch activity (Dinh et al. 2022, You et al. 2005). This mechanism relies on the availability of respective binding sites for both Nkx2-3 and COUP-TFII within the MAdCAM-1 promoter region in HEV-programmed venous endothelial cells. As Prox1 also exerts its LEC-defining activity in venous endothelial cells (Wigle and Oliver 1999), it is tempting to speculate that a similar dual regulation may take place in the spleen, where Nkx2-3 may inhibit the expression of Prox1, while COUP-TFII continues to define venous specification. Indeed, search of promoter database (https://epd.epfl.ch) pinpoints potential bindings sites for both Nkx2-3 and COUP-TFII in the Prox1 promoter region. It remains to be investigated whether Nkx2-3, together with COUP-TFII (thus in a vein-specific manner) may influence Prox1 expression, thereby pivoting the sinus vein endothelium towards LEC fate commitment, in addition to the altered postnatal blood endothelial addressin preference from MAdCAM-1 to PNAd upon lymphocyte accumulation (Mebius et al. 1996, Balázs et al. 2001, Balogh et al. 2007, Zindl et al. 2009).

Concerning the functional consequences of this shift, we have observed a profound defect of extramedullary hematopoiesis along both the erythroid and megakaryocyte lineages, even though phenotypic analyses indicate the presence of non-endothelial CD29+Sca-1+gp38+Thy1.2+CD51+ positive splenic stromal cells which are also present in wild-type mice and are instrumental for RP-associated hematopoiesis (Lim and O'Neill 2019). As the bone marrow erythropoiesis could be augmented following blood loss in both wild-type and Nkx2-3 mutants, the lack of Nkx2-3 is unlikely to cause the failure of hematopoietic stem cells to promote extramedullary hematopoiesis. The RP sinus endothelial cells provide niches for hematopoietic stem cells (Kiel et al. 2005), therefore the deviation of RP sinus endothelium specifics (reflected in their lack of Clever1 expression and IBL-9/2 staining) may be a critical factor in the defective splenic hematopoiesis. Splenic macrophages (including MARCO-positive MZ macrophages, CD169-positive metallophilic macrophages as two main MZ resident macrophages as well as F4/80-positive RP monocytes/macrophages) may also promote extramedullary hematopoiesis in stress responses (Lévesque et al. 2021). As Nkx2-3 mutant spleens have a dearth of these cells (Pabst et al. 1999, Wang et al. 2000), the resulting constitutional anemia and impaired extramedullary hematopoiesis may also be linked to these cells. Moreover, the blocked splenic hematopoiesis may be related to the formed LECs themselves as well, as recent findings indicate that the acquisition of Prox1 represses the hemogenic capacity of endothelial cells (Kazenwadel et al. 2023). Clearly, further studies are needed to isolate various defined stromal subsets from Nkx2-3-deficient and wild-type mice, and compare their composition as well as gene expression characteristics.

In summary, our findings firmly establish the presence of gp38+/CD31+/Prox1+ LECs forming ectopic lymphatic capillaries in the spleens of mice lacking Nkx2-3, which is coupled with a phenotypic shift of RP vasculature lacking sinus-endothelium specific markers Clever1 and IBL-9/2. This shift in RP vascular patterning is associated with defective extramedullary hematopoiesis manifesting as constitutional anemia under steady-state conditions and repressed stress hematopoietic response and defective megakaryocyte expansion, together demonstrating the complex role of Nkx2-3 in shaping the vascular identity and functionality of RP.

In the second part of our work, we studied the presence and distribution of Nkx2-3 homeodomain transcription factor in humans to define its course of expression during aging

and in colorectal malignancies. Our findings demonstrate a heterogeneous expression pattern shared between various endothelial as well as fibroblastic cell subsets, and differential production affected by both aging and malignant transformation.

First, we investigated the colonic expression pattern of Nkx2-3 at various ages from histologically normal samples. Our findings reveal a more pronounced expression in the youngest age group, probably reflecting the organ growth in this period. The bulk of reactivity was observed in the subepithelial region of the colonic lamina propria, where a shared co-expression with mesenchymal stromal markers was detected, including CD34, αSMA and VAP-1.

The endothelial expression of Nkx2-3 may be needed for the maintenance of capillary organization, as its inherited mutation has been demonstrated to be associated with intestinal varices (Kerkhofs et al. 2020). Furthermore, the inhibition of Nkx2-3 in ileal microvascular endothelial cells in vitro led to the reduction of mRNA expression of MAdCAM-1, AKT and VCAM1 surface molecules (Yu et al. 2011). These observations indicate endothelial effects as well as possible communication (membrane-associated or soluble) with other cells, including leukocytes that can contribute to the hitherto unexplored relationship between altered expression of Nkx2-3 and inflammatory bowel diseases (Parkes et al. 2007). The endothelial effects of Nkx2-3 in murine Peyer's patches high endothelial venules involve the transcriptional action of a composite recognition element in the regulatory regions for Nkx2-3 and COUP-TFII proteins, influencing the expression of MAdCAM-1 addressin and St6Gal1 sulfotransferase enzyme defining the addressin's glycosylation characteristics (Dinh et al. 2022). While Nkx2-3 is also expressed in hematopoietic stem cells (HSCs) influencing their homeostasis, in mature leukocytes Nkx2-3 is absent, in agreement with our immunohistochemical observations. Although the absence of Nkx2-3 causes defective development of Peyer's patches and isolated follicles, and suppresses DSS-induced colitis (Kellermayer et al. 2019, Pabst et al. 2000, Wang et al. 2000), this deviation is likely be related to disrupted endothelial specification affecting intestinal immunity.

While the (venous) endothelial expression of Nkx2-3 may contribute to leukocyte homing to the intestines and inflammatory responses, the non-endothelial production of Nkx2-3 within the lamina propria may be more closely related to the differentiation of colonic epithelial cells, via establishing the colonic niche for intestinal epithelial stem cells (IESCs) expressing Lgr5 (Barker et al. 2007, Hsia et al. 2016). In our subsequent analyses aimed at defining the lineage affiliation of Nkx2-3-positive colonic stromal cells, we consistently found that the colonic epithelial cells themselves do not express detectable amounts of Nkx2-3 protein; on the other hand, a significant fraction of the non-endothelial lamina propria stromal cells expressing either CD34, αSMA and/or VAP-1 produce Nkx2-3. This arrangement is similar to that in mice, where the proximity of $Lgr5^+$ IESCs and $CD34^+/gp38^+$ pericryptal mesenchymal cells producing Wnt2b, Gremlin-1 and R-spondin1 appears a main factor in maintaining the homeostasis of IESCs following tissue damage and inflammation (Stzepourginski et al. 2017). The differential representation of $Nkx2\text{-}3^+$ cells amongst the VAP-1-positive and CD34-positive pericryptal compartment, together with the difference between the total Nkx2-3 positive nuclei and the sum of VAP-1 and CD34 expression suggests both a partial overlap for VAP-1 and CD34 (thus the likely existence of $VAP\text{-}1^+/CD34^-$ cells) and the presence of $VAP\text{-}1^-/CD34^-$ cells amongst the pericryptal Nkx2-3-positive cells. To clarify the distribution of these presumed stromal subsets, the combination of CD31, CD34 with VAP-1 as surface markers correlated with Nkx2-3 expression may offer a possible approach to identify putative intestinal myofibroblastic cells for further analyses, including their support functions for epithelial differentiation of IESCs. This approach may also confirm the existence of a putative "triple negative" (i. e. CD34/VAP-1/αSMA-negative) Nkx2-3-positive subset demonstrated in our combined

immunohistochemical labeling that we assume as a minor population. Our immunohistochemical results demonstrating the lack of clear lineage-restricted expression of Nkx2-3 are in line with recent scRNAseq analyses revealing a wide range of mesenchymal cells transcribing Nkx2-3, including (but not limited to) myofibroblasts, ADAMDEC$^{+}$ stromal cells, CCL11$^{+}$ stromal cells, CH25H$^{+}$ stromal cells and others (Elmentaite et al. 2021) (www.gutcellatlas.org]. Within the endothelial compartment, NKX2-3 was transcribed in cycling (Ki67$^{+}$) endothelial cells and capillary endothelium, but not in lymphatic endothelium.

Finally, in our immunohistochemical analysis of NKX2-3 expression in polyps and CRC samples we found that NKX2-3 is downregulated in the connective tissue. This raises the question whether the expansion of cancer cells may alter the transcriptional profile of cancer-associated fibroblasts (CAFs), or the initial reduction of NKX2-3 may create a permissive environment for the emergence of CRC. The observed reduction of NKX2-3 suggests either reduced production by the resident stromal cells, or their partial replacement by cells originally lacking NKX2-3 production. Our earlier findings on the enhanced epithelial proliferation and intestinal regeneration in mice lacking Nkx2-3 in DSS-induced colitis model (Kellermayer et al. 2019) can be reconciled with the negative relationship between NKX2-3 expression and epithelial cell expansion in human colon. It remains to be investigated whether the lack of Nkx2-3 enhances the propensity for colorectal cancers in these mice. As suggested above, exploiting the possible combination of CD31 (to specify endothelial cells) with CD34 and VAP-1 (as shared endothelial/mesenchymal markers) for cell sorting may also provide further data on the altered production of NKX2-3 between normal and CRC or polyp samples from humans. It is yet unknown whether various CRC-associated mutations (KRAS, NRAS and BRAF) (Bożyk et al. 2022) or FAP production (Kalaei et al. 2023) may modify or correlate with the expression of NKX2-3. In addition, follow-up studies of samples taken from various stages of cancer progression and therapeutic response, and larger cohort may further pinpoint differential representation of FAP-positive CAF compartment with various degrees of NKX2-3 expression. As the expression of NKX2-3 in in the non-invasive adenomatous polyps (with preserved stromal composition) is already reduced, it is likely that the downregulation of NKX2-3 is associated with creating a permissive condition for subsequent transformation and malignant expansion of cancer cells in CRC. Further studies with lineage-specific targeted inactivation of Nkx2-3 in mice may provide important clues on the role of this mesenchymal transcription factor in the propagation of epithelium-derived colonic malignancies.

## 6. Short summary of new results

- **What is the origin of LYVE-1 positive sac-like formations in the spleen in absence of Nkx2-3?**
  - -We detected an extensive Prox1-positive network forming branches and anastomoses in the spleens of Prox1$^{GFP}$-Nkx2-3 mutant mice, which was not present in the spleens of Prox1GFP-Nkx2-3 heterozygous mice.
  - -We confirmed the lymphatic endothelial (LEC) origin of the ectopic Prox-1$^{+}$ capillaries in the spleens of Prox1$^{GFP}$-Nkx2-3 mutant mice by flow cytometry.

- **Other than the known structural abnormalities, what are the effects of Nkx2-3 deficiency on splenic red pulp sinus endothelial cells?**
  - - In samples from wild-type mice, Clever1 expression to a large extent overlaps with IBL-9/2 reactivity. In the absence of Nkx2-3, splenic red pulp sinus endothelial cells lack IBL-9/2 staining and Clever1 protein expression. Spleen samples from Stab1$^{-/-}$ mice labeled with IBL-9/2 antibody revealed an intact pattern of the red pulp sinusoidal network.

- **What is the effect of Nkx2-3 deficiency on extramedullary red blood cell formation?**
  - -Spleens from Nkx2-3-deficient mice show stromal and vascular CD29$^{+}$/VCAM-1$^{+}$ cells, and the red pulp shows a fragmented structure without a clear red pulp-white pulp boundary.
  - -In untreated Nkx2-3 KO mice, chronic anaemia is present and EPO levels are significantly higher than in the control group, which is not sufficient to maintain normal hematocrit levels. After treatment, EPO levels in Nkx2-3 KO mice were significantly higher than in treated BALB/c mice, but not sufficient to induce stress erythropoiesis.
  - -In the absence of Nkx2-3 in the spleen, TER119+ erythrocytes can be detected, and the frequency and number of erythroid cells are also lower compared to controls. After treatment, the number of erythroid cells and the TER-119+ area do not increase, but CD29+ extravascular reticular cells are present. In the absence of Nkx2-3, the spleen is unable to increase its erythropoietic activity, suggesting an impairment of tissue-specific erythropoietic function.
  - -Following treatment, both wild-type and Nkx2-3 KO mice show increased bone marrow hematopoiesis and it is hypothesized that the lack of Nkx2-3 leads to a decrease in hematopoiesis through damage to non-hematopoietic stem cells.

- **Does impaired extramedullary haematopoiesis in the absence of Nkx2-3 affect the incidence and expansion of megakaryocytes in the spleen?**
  - -In untreated BALB/c mice, megakaryocytes were found in the subcapsular layer and deeper regions of the red pulp as single cells or doublets, whereas in the absence of Nkx2-3 they were only scattered.
  - -TPO agonist treatment leads to a significant increase in platelet counts in wild-type spleen, whereas in the absence of Nkx2-3 there is only a small increase.
  - - The spleens of Nkx2-3 deficient mice have a reduced number of megakaryocytes and are unable to increase the number of this population upon TPO agonist treatment, compared to BALB/c mice.

- **Are there any differences in the expression of the NKX2-3 transcription factor in morphologically intact human colon tissue samples of different age groups?**
  - The NKX2-3 protein can be detected in human colorectal tissue samples by immunohistochemistry.
  - NKX2-3 is homogeneously distributed in the tunica mucosa and tunica muscularis layers.
  - The expression level and H-score of NKX2-3 protein differs in different age groups.
  - The expression of NKX2-3 is highest in the youngest age group.

- **Which stromal element(s) are associated with NKX2-3 protein expression in human colon?**
  - The vast majority of non-vascular $CD34^+$ cells in the pericrypt region are $NKX2\text{-}3^+$.
  - The majority of $VAP\text{-}1^+$ cells in the pericryptal region also express NKX2-3 protein.
  - Among $NKX2\text{-}3^+$ cells, there are proportionally more $VAP\text{-}1^+$ cells than $CD34^+$. VAP-1 expression is more closely associated with NKX2-3 production than CD34.
  - The overwhelming majority of $NKX2\text{-}3^+$ cells are CD34/VAP-1/αSMA positive, however, there are present $NKX2\text{-}3^+$ cells that do not display any of the above phenotypic characteristics.
  - The majority of histopathologically detectable $NKX2\text{-}3^+$ cells belong to the pericryptal myofibroblast cell line.

- **Does the expression of NKX2-3 change in pre-malignant and malignant human colon lesions?**
  - $NKX2\text{-}3^+$ cells were detected in the lamina propria/stroma layer in both colonic polyp and adenocarcinoma samples,
  - The morphologically intact control group had the highest percentage of $NKX2\text{-}3^+$ nuclei, a significant difference compared to the morphologically intact-polyp and morphologically intact-tumour groups.
  - Weakly staining NKX2-3 cells are present in the highest numbers in both polyp and tumour samples, which is associated with a significant reduction in the number of moderately and strongly staining $NKX2\text{-}3^+$ nuclei.
  - NKX2-3 is undetectable in the epithelial cell layer in samples containing tumour and polyposis lesions.

# 7. List of publications and lectures

### Publication the thesis is based on

**Gábris, F**., G. Kiss, B. Szirmay, Á. Szomor, G. Berta, Z. Jakus, Z. Kellermayer, and P. Balogh. 2023. Absence of Nkx2-3 induces ectopic lymphatic endothelial differentiation associated with impaired extramedullary stress hematopoiesis in the spleen. *Front Cell Dev Biol* 11: 1170389.
**IF:5,5**

**Gábris, F**., B. Kajtár, Z. Kellermayer, and P. Balogh. 2023. Quantitative analysis of Nkx2-3 expression in human colon – an immunohistochemical study. *J Histochem Cytochem:* 221554231217336

### Other related publications

Vojkovics, D., Z. Kellermayer, **F. Gábris**, A. Schippers, N. Wagner, G. Berta, K. Farkas, and P. Balogh. 2019. Differential Effects of the Absence of Nkx2-3 and MAdCAM-1 on the Distribution of Intestinal Type 3 Innate Lymphoid Cells and Postnatal SILT Formation in Mice. *Front Immunol* 10: 366
**IF:5,085**

Dinh, T. T., M. Xiang, A. Rajaraman, Y. Wang, N. Salazar, Y. Zhu, W. Roper, S. Rhee, K. Brulois, E. O'Hara, H. Kiefel, T. M. Dinh, Y. Bi, D. Gonzalez, E. P. Bao, K. Red-Horse, P. Balogh, **F. Gábris**, B. Gaszner, G. Berta, J. Pan, and E. C. Butcher. 2022. An NKX-COUP-TFII morphogenetic code directs mucosal endothelial addressin expression. *Nat Commun* 13: 7448.
**IF:16,6**

### Publication not directly related to the thesis

**Gábris Fanni**, Xinkai Jia, Gajdócsi Erzsébet Emília, Kellermayer Zoltán, Balogh Péter. 2020. A nyirokcsomóstroma immunológiai szerepe – többen, többet. *Immunológiai szemle*

Jia, X., G. Berta, **F. Gábris**, Z. Kellermayer, and P. Balogh. 2020. Role of adipose-associated lymphoid tissues in the immunological homeostasis of the serosal surface. *Immunol Lett* 228: 135-141.
**IF:3,685**

Jia, X., **F. Gábris**, Ó. Jacobsen, G. Bedics, B. Botz, Z. Helyes, Z. Kellermayer, D. Vojkovics, G. Berta, N. Nagy, Z. Jakus, and P. Balogh. 2020. Foliate Lymphoid Aggregates as Novel Forms of Serous Lymphocyte Entry Sites of Peritoneal B Cells and High-Grade B Cell Lymphomas. *J Immunol* 204: 23-36
**IF:5,422**

Khanfar, E., K. Olasz, **F. Gábris**, E. Gajdócsi, B. Botz, T. Kiss, R. Kugyelka, T. Berki, P. Balogh, and F. Boldizsár. 2020. Ameliorated Autoimmune Arthritis and Impaired B Cell Receptor-Mediated $Ca^{2+}$ Influx in Nkx2-3 Knock-out Mice. *Int J Mol Sci* 21.
**IF:5,924**

## 8. 8. Acknowledgements

I would like to thank all the people who helped me to complete my PhD.

First of all, I would like to thank my supervisors Prof. Dr. Péter Balogh and Dr. Zoltán Kellermayer for their support and guidance during my years as a research student and later as a PhD student.

I would like to thank all the members of the Department of Immunology and Biotechnology for their help and support.

I would like to thank Dr. Béla Kajtár, who helped me in the human studies with sample selection and analysis.

I would like to thank Dr. Gergely Berta for his help in preparing confocal microscopy images.

I am grateful to my family and friends for their support and encouragement throughout my work.

My work was made possible with the following grants:
OTKA- #K108429
EFOP-3.6.1-16-2016-00004
FIKP- Programme for Institutional Excellence in Higher Education (FIKP II)
TKP-2020-4.1.1.-TKP2020 György Romhányi College