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Generation and Analysis of Serine Protease Inhibitor Kazal Type 3-Cre Driver Mice

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Abstract: Serine protease inhibitor Kazal type 1 (*SPINK1*; mouse homologue *Spink3*) was initially discovered as a trypsin-specific inhibitor in the pancreas. However, previous studies have suggested that *SPINK1/Spink3* is expressed in a wide range of normal tissues and tumors, although precise characterization of its gene expression has not been described in adulthood. To further analyze *Spink3* expression, we generated two mouse lines in which either *lacZ* or Cre recombinase genes were inserted into the *Spink3* locus by Cre-*loxP* technology. In *Spink3*^{lacZ} mice, β-galactosidase activity was found in acinar cells of the pancreas and kidney, as well as epithelial cells of the bronchus in the lung, but not in the gastrointestinal tract or liver. *Spink3*^{cre} knock-in mice were crossed with Rosa26 reporter (R26R) mice to monitor Spink3 promoter activity. In *Spink3*^{cre};R26R mice, β-galactosidase activity was found in acinar cells of the pancreas, kidney, lung, and a small proportion of cells in the gastrointestinal tract and liver. These data suggest that Spink3 is widely expressed in endoderm-derived tissues, and that *Spink3*^{cre} knock-in mice are a useful tool for establishment of a conditional knockout mice to analyze Spink3 function not only in normal tissues, but also in tumors that express *SPINK1/Spink3*.
Key words: SPINK1, Spink3, *Spink3*^{cre} mice, *Spink3*^{lacZ} mice

Introduction

Serine protease inhibitor Kazal type 1 (*SPINK1*), also known as pancreatic secretory trypsin inhibitor (*PSTI*), was originally isolated from the pancreas [5]. In mice, the homologous gene is designated as *Spink3*(serine protease inhibitor Kazal type 3) [10]. SPINK1 is produced in acinar cells of the exocrine pancreas, and is packaged with digestive enzymes into granules that are secreted into the pancreatic duct [11]. SPINK1/Spink3 covalently binds to erroneously activated trypsin in the pancreas to form an inactive and stable complex to prevent acute pancreatitis, a major inflammatory disorder of the pancreas [11]. The *SPINK1* gene is a candidate gene of hereditary pancreatitis, although its pathogenesis is unknown [18]. In addition to the pancreas,

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SPINK1 has been subsequently identified in mucus-producing cells throughout the gastrointestinal tract [4] and in a range of other tissues including the lung, liver, kidney, ovary, breast, and the collecting tubules and transitional epithelium of the renal pelvis in humans [7]. Interestingly, SPINK1 was isolated from urine of ovarian cancer patients, and reported as a tumor-associated trypsin inhibitor (TATI) [16]. Increased expression of SPINK1 protein has been reported in various cancers such as lung, colon, liver, and prostate cancers, and is associated with poor survival of patients [11]. However, the role of SPINK1/TATI in normal tissues and malignant tumors is unknown. We previously showed that excessive autophagy is induced in *Spink3* knockout mice, and that Spink3 is essential to maintain the exocrine integrity of the pancreas and possibly acts as a growth factor for pancreatic acinar cells [10]. *Spink3* knockout mice die within 2 weeks after birth, making it difficult to monitor the long-term effects of *Spink3* deficiency.

To gain an insight into its function, we previously analyzed *Spink3* expression profiles by *in situ* hybridization and *lacZ* expression during embryonic development of a *Spink3* knock-in ($Spink3^{lacZ}$) mouse, in which the *lacZ* gene had been inserted into the *Spink3* locus [17]. The *lacZ* gene was first observed in the foregut, midgut, and hindgut at 9.5 days post-coitus (dpc). In the pancreas, *Spink3* mRNA was detected at 11.5 dpc prior to formation of the typical shape of the exocrine structure of the pancreas. After differentiation of the intestinal tract, *lacZ* expression was observed in the large intestine at 11.5 dpc, followed by expression in the small intestine at 13.5 dpc before the appearance of digestive enzymes in the intestine. *Spink3* was also expressed in other tissues including mesonephric tubules and the urogenital ridge, the genital swelling, ductus epididymis, and seminal vesicles. These data suggest that Spink3 plays important roles in proliferation and/or differentiation of various cell types during development [17].

As the *Spink3* gene is expressed in several tissues, the Cre driver mouse in which the *Cre* gene is expressed under the control of Spink3 promoter/enhancer will be a useful tool to produce conditional knockout mice. In this study, we generated the $Spink3^{cre}$ mouse, in which the *Cre* gene had been inserted into the mouse *Spink3* locus using the exchangeable gene targeting method [1]. Then we compared the expression pattern between $Spink3^{lacZ}$ and $Spink3^{cre}$;R26R adult mice by X-gal staining. We showed that $Spink3^{cre}$ mice are useful and unique tool for generating various conditional knockout mice.

Methods

Mice

In all experiments, 8-week-old mice were used. C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). All mouse experiments were performed in accordance with the Declaration of Helsinki and were approved by the Kumamoto University Ethics Committee for Animal Experiments.

Northern and Western blot analyses

Total RNA was isolated from each mouse organ with Sepasol (Nacalai Tesque, Kyoto, Japan). For Northern blot analysis, 10 μg of RNA was fractionated by 1.4% agarose gel electrophoresis. Filter-bound RNA was sequentially hybridized with a digoxigenin-labeled RNA probe (full-length *Spink3* cDNA). For Western blot analysis, each organ was homogenized in lysate buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.1% Triton X-100, 10% glycerol, 1 mmol/L NaF, 2 mmol/L sodium orthovanadate, 1 mmol/L ethylenediaminetetraacetic acid, and protease inhibitor cocktail [1:100 dilution; Sigma-Aldrich, Tokyo, Japan]). Protein extracts (15 μg per lane) were applied to 5–20% gradient polyacrylamide gels (E-T/R/D520L, ATTO, Tokyo, Japan) for electrophoresis, and then transferred to an Immobilon polyvinylidene difluoride filter (Millipore, Darmstadt, Germany). Primary rabbit antibodies against the following antigens were used at the indicated dilutions: Spink3 (1:1000; Transgenic Inc., Kobe, Japan) and actin (1:1000; Sigma-Aldrich). An anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (1:2500 dilution; Amersham, Buckinghamshire, UK) was used for detection.

Generation of the $Spink3^{lacZ}$ mouse strains

Generation of $Spink3^{lacZ}$ mice has been described previously [17].

Construction of the replacement vector loxJTZ17-NLS-Cre-lox2272-MC1-DT-A

The *lox*JTZ17 [3] fragment was inserted at the 5′ end of nuclear localization signal (NLS)-Cre-poly (A) (pA) fragment. A phosphoglycerate kinase-1 (PGK) promoter-driven puromycin resistance gene (PAC)-pA cassette was

inserted at the 3′ end. The *lox*2272 sequence [1] was introduced into the multiple cloning site at the 3′ end of the PGK-PAC-pA cassette. Finally, a polyoma enhancer/herpes simplex virus thymidine kinase promoter (MC1)-driven diphtheria toxin A chain gene (DT-A)-pA cassette, which is used for negative selection, was cloned to the 3′ end of the *lox*2272 fragment, resulting in construction of the *lox*JTZ17-NLS-Cre-*lox*2272-MC1-DT-A plasmid [2]. Thus, this plasmid contained *lox*JTZ17, Cre fused to a SV40 large T antigen NLS and an intron, which include splice donor (SD) and splice acceptor (SA), and a pA signal derived from SV40, *lox*2272, and MC1-DT-A-pA.

Establishment of the Spink3^{cre} embryonic stem (ES) cell line

The *Spink3*$^{+/-}$ ES cell line was obtained by knocking-in the PGK-neomycin resistance gene fragment flanked by mutant lox sites, *lox*71 and *lox*2272 into the exon 1 of *Spink3* gene (Fig. 2A) and maintained as described previously [10]. The *Cre* expression vector, pCAGGS-Cre, was constructed by inserting the Cre fragment from pBS185 (Life Technologies, Tokyo, Japan) into pCAGGS [8].

The *Spink3*$^{+/-}$ ES cells (1×10^7) were co-electroporated with both pCAGGS-Cre and *lox*JTZ17-NLS-Cre-*lox*2272-MC1-DT-A vetcors (each 20 μg) using a Bio-Rad Gene Pulser (Bio-Rad, Tokyo, Japan), and then cultured in medium supplemented with 2 μg/ml puromycin (Sigma-Aldrich) for 48 h. Selection was performed for 5 days, and then colonies were picked, transferred to 48-well plates, and expanded for cryopreservation. The puromycin-resistant colonies were analyzed by Southern blot and polymerase chain reaction (PCR) to select ES cell lines with successful integration of *cre*. DNA from the puromycin-resistant colonies was digested with *Pst*I and was used to detect replaced alleles by Cre probe. PCR analysis to detect the replaced alleles, was performed with the following primers: S1,5′-agttcttctggcttttgcaccc-3′, and A1, 5′atagtcgacttcttctttg-gttttcggg-3′′. This primer set is expected to give PCR products with a size of 1.2kbp. PCR consisted of an initial denaturation cycle at 94°C for 5 min, followed by the 30 cycles consisted of denaturation at 94°C for 20 s, annealing at 55°C for 30 s, and elongation at 68°C for 2 min. Five replaced ES cell clones were obtained among 30 puromycin-resistant clones analyzed (Fig. 2B). Positive clones were aggregated with ICR (Sea:ICR, purchased from Kyudo Co., Ltd., Tosu, Japan) morula according to a protocol described previously [10]. Germ-line transmission was observed in two mice after mating with C57BL/6J mice.

X-gal staining for β-galactosidase (β-gal) activity

We dissected tissues from *Spink3*$^{+/+}$;R26R, as a control, *Spink3*^{lacZ}, and *Spink3*^{cre}; R26R mice at 8 weeks of age. For whole organ staining, the method has been described previously [17]. For section staining, optimum cutting temperature (OCT) (Sakura, Tokyo, Japan)-embedded tissues were cut using a cryostat to prepare 60 μm-thick sections. The sections were fixed with 1% formaldehyde, 0.2% glutaraldehyde and 0.02% NP-40 for 30–60 min at 4°C. Fixed specimens were rinsed with phosphate buffered saline (PBS) three times, and then 0.1% Triton X-100 in PBS three times. The specimens were then immediately incubated at 37°C for 12–16 h, depending on the rate of the color reaction, in an X-gal staining solution containing 5 mM potassium hexacyanoferrate II, 50 mM potassium hexacyanoferrate III, 2 mM $MgCl_2$, and 0.5% (w/v) X-gal (4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside) (Nacalai Tesque) in PBS [17]. Finally, the samples were washed twice with PBS and post-fixed in 15% formalin.

Results and Discussion

We first analyzed *Spink3* expression at both mRNA and protein levels in 8-week-old C57BL/6J male mice. In Northern blot analysis, *Spink3* mRNA was strongly expressed in the pancreas, small intestine, and kidney, but not in the brain, heart, lung, liver, or spleen (Fig. 1). In Western blot analysis, Spink3 was expressed strongly in the pancreas, and slightly in the small intestine, large intestine, and kidney. In the brain, heart, lung, liver, and spleen, there was no *Spink3* expression (Fig. 1).

To study cell type-specific expression of *Spink3*, we generated knock-in mice expressing *lacZ* (*Spink3*^{lacZ}) or the Cre recombinase gene (*Spink3*^{cre}) driven by the endogenous *Spink3* promoter by Cre-*loxP* recombination technology (Fig. 2A). These two heterozygous mouse lines appeared to be normal and were fertile. *Spink3*^{cre} knock-in mice were crossed with Rosa26 reporter (R26R) mice [15] to produce *Spink3*^{cre};R26R mice with which Spink3 promoter activity can be monitored. We detected the activity of β-gal by X-gal staining. Whole mount staining of *Spink3*^{lacZ} mice showed β-gal activity only

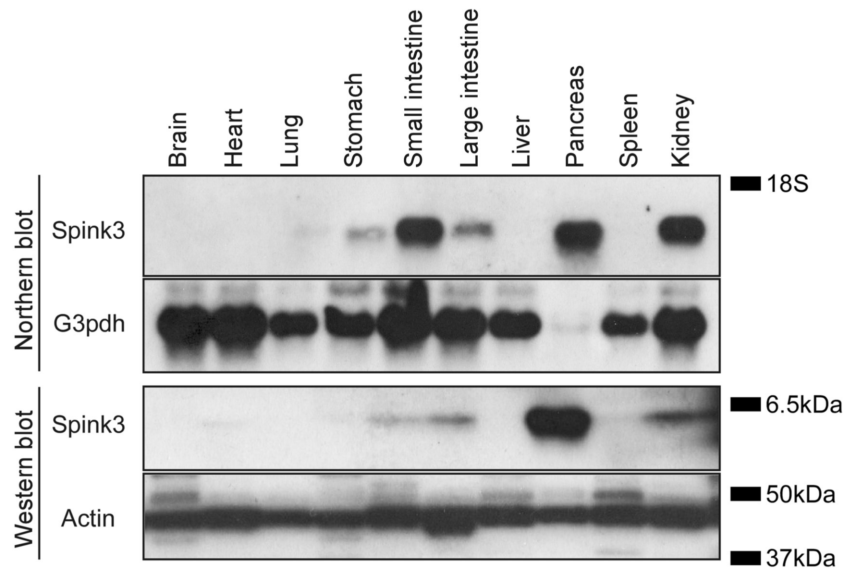
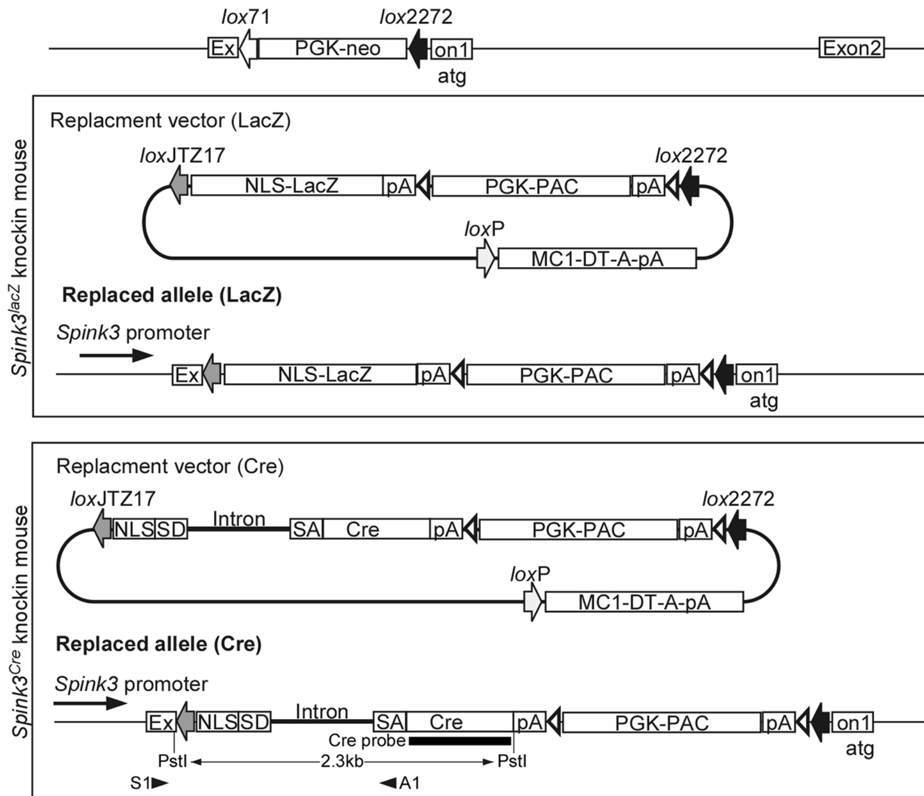


Fig. 1. Expression pattern of Spink3 in the adult mouse. Northern (upper panel) and Western (lower panel) blot analysis of Spink3 in an 8-week-old C57BL/6J mouse. 18S; the small ribosomal subunit.

in the pancreas, but not in the liver (Fig. 3A). In contrast, *Spink3*^{cre}; R26R mice showed β-gal activity not only in the pancreas, but also in some liver cells in a mosaic pattern. Pancreatic acinar cells (exocrine pancreas), which are the primary source of digestive enzymes, account for nearly 90% of the pancreatic mass. Consistent with the known distribution of endogenous *Spink3* expression, X-gal staining in *Spink3*^{lacZ} mice at 8-week-old was exclusively localized to pancreatic acinar cells, and not in duct or endocrine islets cells (Fig. 3B). In the pancreas of *Spink3*^{cre}; R26R mice, X-gal staining was localized to pancreatic acinar cells, but not in duct cells. Interestingly, there were some X-gal positive endocrine cells in *Spink3*^{cre}; R26R mice (Fig. 3B). This observation and our previous data [17] indicated that *Spink3* was expressed not only in immature and mature acinar cells, but also in some immature endocrine cells. In the liver, there were some X-gal-positive liver cells in *Spink3*^{cre}; R26R mice, but not in *Spink3*^{lacZ} mice. Although *Spink3* was not detected in the liver under physiological conditions, Kobayashi *et al.* reported that the concentration of human SPINK1 protein is higher in the livers of adult-onset type II citrullinemia patients, which is caused by a deficiency of argininosuccinate synthetase protein in the liver, compared with that in the normal control [6]. Ohmachi *et al.* reported that *SPINK1* mRNA is highly expressed in hepatic cancer [9]. In addition, SPINK1 has been suggested to be an acute-phase reactant in humans, which is induced by inflammatory cytokines such as interleukin (IL)-1 and IL-6 [19]. Thus, *Spink3* expression may be induced in the liver by inflammation or liver disease. In addition, we previously showed that *Spink3* was widely expressed in digestive organs during development [17]. Taken together, it was possible that *Spink3* was expressed in some hepatic cells during embryonic development.

Previous reports have shown that SPINK1 protein and mRNA are expressed in mucus-producing cells of the normal gastrointestinal tract [4, 7, 13]. SPINK1 may act as a protease inhibitor to protect epithelial cells from some proteases. Our previous data also showed that *lacZ* is strongly expressed in epithelial cells of both the small and large intestines in *Spink3*^{lacZ} mice from 13.5 to 17.5 dpc [17]. However, in this study, β-gal activity was not detected in the gastrointestinal tracts of adult *Spink3*^{lacZ} mice (Figs. 4A and B). On the other hand, in adult *Spink3*^{cre}; R26R mice, spotty β-gal activity was detected in the digestive tract from the stomach to the large intestine (Figs. 4A and B). β-gal expression in adulthood can be explained as follows. In *Spink3*^{cre}; R26R mice, Cre is expressed during embryonic development, leading to β-gal expression. Once the recombination occurs, β-gal gene expression continues under the R26 promoter.

In the kidney, *Spink3* mRNA is expressed mainly



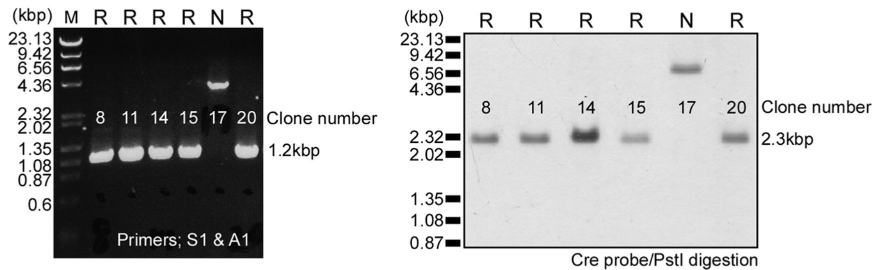


Fig. 2. Targeting strategy for lacZ and Cre knock-in mice. (A)*LacZ* and Cre recombinase genes were inserted into the *Spink3* locus by Cre-l*ox*P technology. ES cells carrying *Spink3* targeted allele were first created by knocked-in the PGK-neo expression cassette into the exon 1 of *Spink3* gene. These ES cells were then transfected with replacement vector for lacZ or Cre together with *Cre* expression vector. Closed triangles are PCR primers (S1 and A1) for detecting the Cre replaced clones. Closed box indicate Cre probe for Southern blot analysis. *Pst*I-digested genomic DNA from ES cells was used. Open triangles are FLP recombination target (FRT) sites. SD; splice donor, and SA; splice acceptor. (B) PCR (left panel) and Southern blot (right panel) analysis of DNA from Cre replaced clones. R; replaced clone, N; not replaced clone, M; size marker.

within the medulla area and in a portion of the cortex during development [17]. In adult mice, β-gal activity is detected in the outer zone of the medulla. In addition, we detected β-gal activity in the cortex of the kidney in *Spink3*^{cre}; R26R mice (Fig. 5). Although *Spink3* is expressed from the embryonic stage to adulthood in the kidney, the role of Spink3 is unknown. More precise histological analysis of *Spink3* expression needs to be

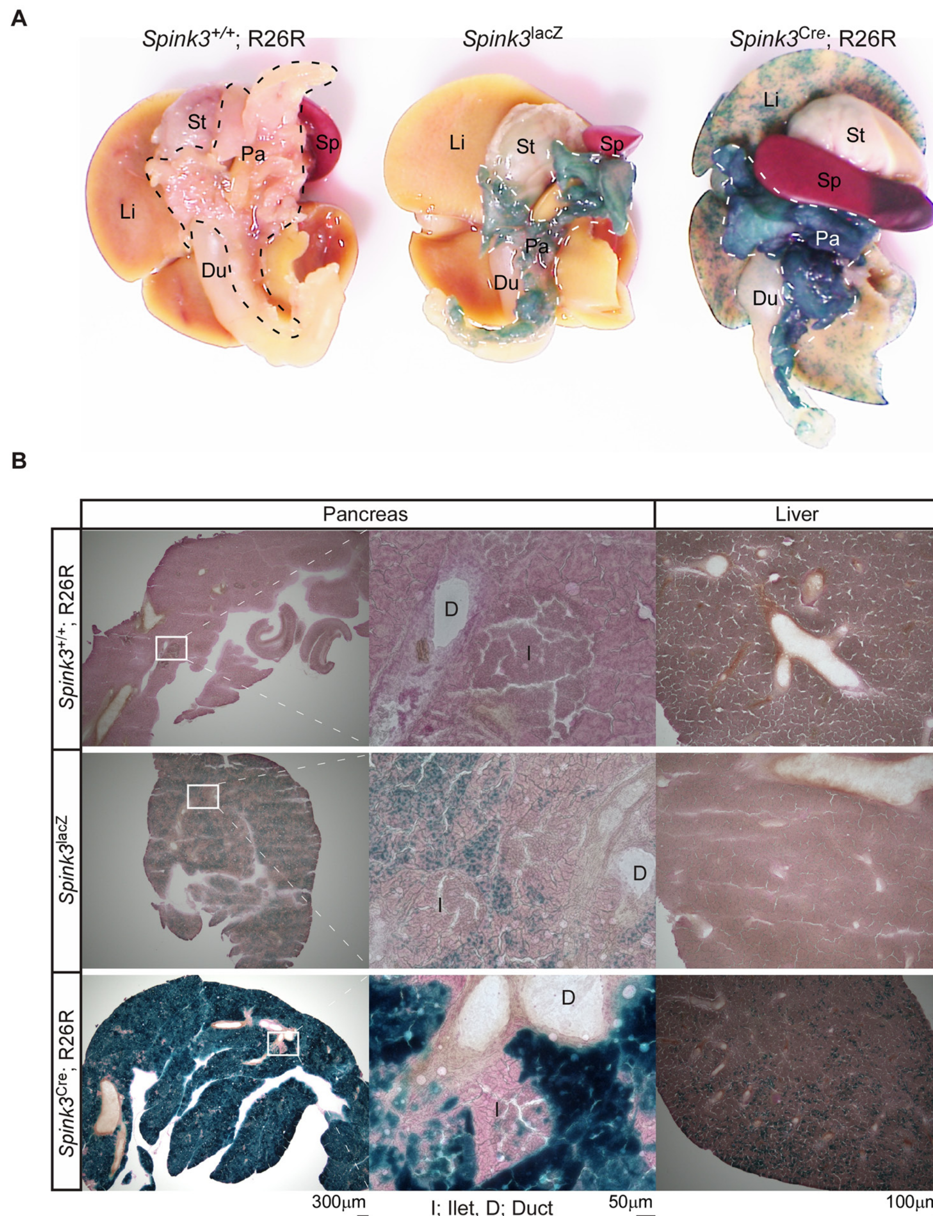


Fig. 3. β-gal activity of the pancreas and liver**.** Whole mount (A) and tissue section (B) X-gal staining of *Spink3*$^{+/+}$;R26R, *Spink3*^{lacZ}*,* and *Spink3*^{cre};R26R mice. Dashed line indicates the pancreas (Pa). Li; liver, St; stomach, Du; Duodenum, Sp; spleen.

performed using specific molecular markers to analyze the role of SPINK1/Spink3 in the kidney.

The expression and role of *SPINK1/Spink3* have never been examined in the lung. We were unable to detect discernible *lacZ* expression or *Spink3* mRNA in the lung. Northern and Western blot analyses revealed that *Spink3* mRNA or protein was not expressed in the adult mouse lung (see Fig. 1). However, a low level of β-gal activity was detected only in the epithelium of the trachea, but not in the lung acini of *Spink3*^{lacZ} mice (Figs. 6A and B). In *Spink3*^{cre}; R26R mice, β-gal activity was detected not only in epithelial cells of the trachea, but also in lung acini.

It has been reported that *SPINK1* is expressed not only in normal tissues/organs, such as the alimentary tract and kidney in addition to the pancreas, but also in malignant tumors of the digestive tract as well as in gynecological malignancies. However, the role of SPINK1

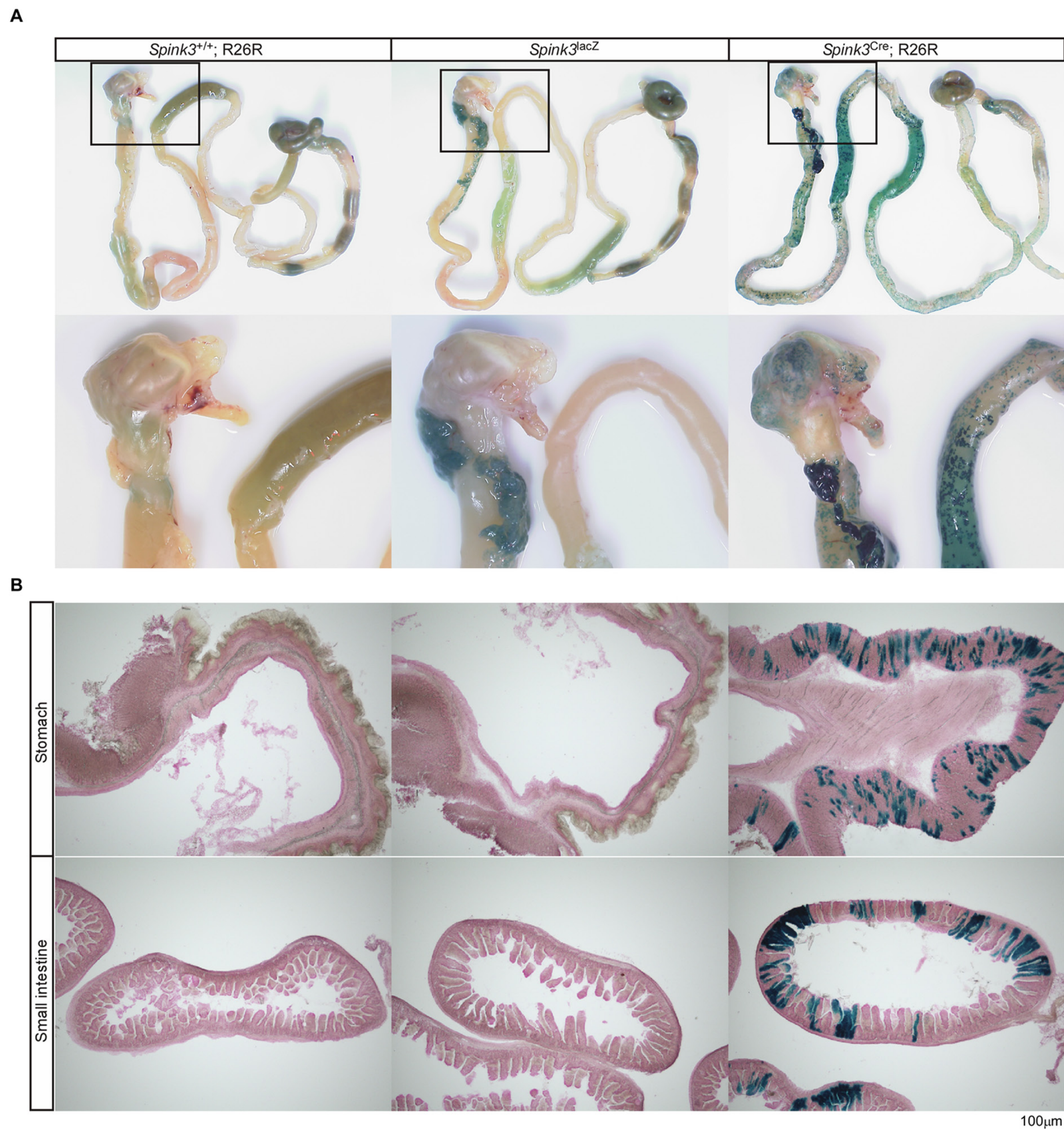


Fig. 4. β-gal activity in the gastrointestinal tract. β-gal activity was not detected in the gastrointestinal tracts of *Spink3*$^{+/+}$;R26R (control) or *Spink3*^{lacZ} mice (whole staining: A, section staining: B). However, in *Spink3*^{cre}; R26R mice, β-gal activity was detected in both crypt and tip compartments of the small intestine.

in these tissues is unclear. It is interesting that SPINK1 is structurally similar to epidermal growth factor (EGF) [14] in terms of the extracellular structure of the EGF receptor. In addition, SPINK1 can bind to the EGF receptor to activate its downstream targets [12]. These results suggest the possibility that SPINK1 participates in specialization and proliferation of cells in addition to its role as a trypsin inhibitor. *Spink3*^{cre} mice will be a unique tool for production of conditional knockout mice to analyze Spink3 function not only in normal tissues/organs but also in tumors that express Spink3.

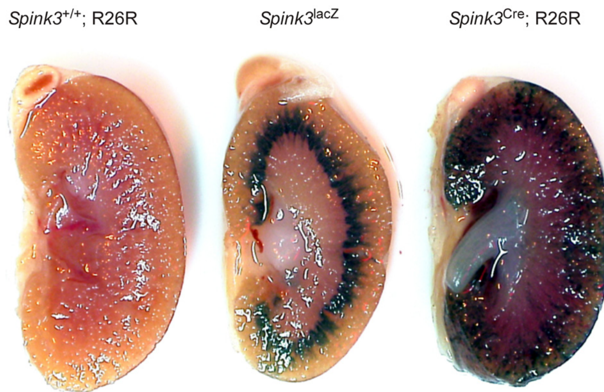


Fig. 5. β-gal activity in the kidney. In the kidney, β-gal activity was detected in the outer zone of the medulla in *Spink3*^{lacZ} mice. β-gal activity was also detected in the cortex of the kidney in*Spink3*^{cre};R26R mice.

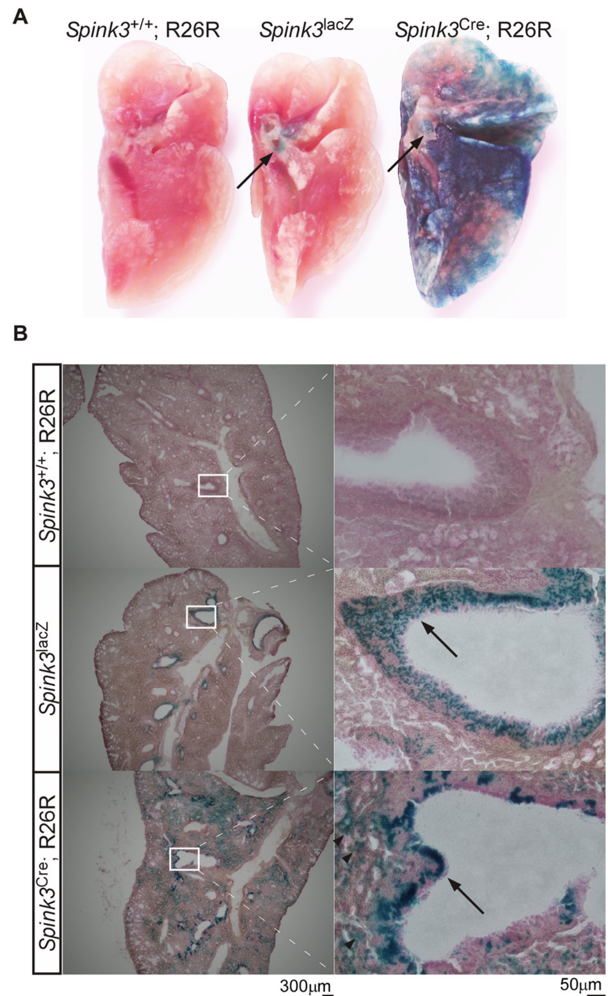


Fig. 6. β-gal activity of the lung. In the lung, β-gal activity was detected only in the epithelium of the trachea, but not in the lung acini of *Spink3*^{lacZ} mice. In *Spink3*^{Cre};R26R mice, β-gal activity was detected not only in the epithelial cells of the trachea, but also in lung acini (whole staining: A; section staining: B). Arrows and arrow heads indicate trachea and lung acini, respectively.

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