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

Kazuya SAKATA1,2)*, Masaki OHMURAYA1)*, Kimi ARAKI1), Chigure SUZUKI3), Satoshi IDA1,2), Daisuke HASHIMOTO2), Jung WANG4), Yasuo UCHIYAMA3), Hideo BABA2), and Ken-ichi YAMAMURA1)

1) Institute of Resource Development and Analysis, Kumamoto University, 2–2–1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan
2) Department of Gastroenterological Surgery, Kumamoto University, 1–1–1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan
3) Department of Cell Biology and Neurosciences, Juntendo University Graduate School of Medicine, 2–1–1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
4) Department of Pathophysiology, College of Basic Medical Sciences, Dalian Medical University, 9 West Section, South Road, Lü Shun, Dalian 116044, P. R. China

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 Spink3lacZ 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. Spink3cre knock-in mice were crossed with Rosa26 reporter (R26R) mice to monitor Spink3 promoter activity. In Spink3cre;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 Spink3cre 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, Spink3cre mice, Spink3lacZ 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,
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 (Spink3lacZ) 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 Spink3cre 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 Spink3lacZ and Spink3cre;R26R adult mice by X-gal staining. We showed that Spink3cre 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/rd520L, 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 Spink3lacZ mouse strains

Generation of Spink3lacZ mice has been described previously [17].

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

The loxJTZ17 [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. Thelox2272 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 thelox2272 fragment, resulting in construction of theloxJ71-NLS-Cre-lox2272-MC1-DT-A plasmid [2]. Thus, this plasmid containedloxJ71, 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,lox2272, and MC1-DT-A-pA.

Establishment of the Spink3cre embryonic stem (ES) cell line

The Spink3cre ES cell line was obtained by knocking-in the PGK- neomycin resistance gene fragment flanked by mutantlox sites,lox71 andlox2272 into the exon 1 ofSpink3 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 Spink3cre ES cells (1×10^7) were co-electroporated with both pCAGGS-Cre andloxJ71-NLS-Cre-lox2272-MC1-DT-A vectors (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 ofcre. DNA from the puromycin-resistant colonies was digested withPstIand was used to detect replaced alleles by Cre probe. PCR analysis to detect the replaced alleles, was performed with the following primers: S1, 5’-gttcttcggg-3’, and A1, 5’atagtcgacttcttctttggc-3’. This primer set is expected to give PCR products with a size of 1.2kb. 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, pur- chased 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, Spink3lacZ, and Spink3cre; 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 hexacyano- ferrate II, 50 mM potassium hexacyanoferrate III, 2 mM MgCl2, 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 ofSpink3, we generated knock-in mice expressinglacZ (Spink3lacZ) or the Cre recombinase gene (Spink3cre) driven by the endogenousSpink3 promoter byCre-loxP recombination technology (Fig. 2A). These two heterozygous mouse lines appeared to be normal and were fertile. Spink3cre knock-in mice were crossed with Rosa26 reporter (R26R) mice [15] to produce Spink3cre;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 Spink3lacZ mice showed β-gal activity only
in the pancreas, but not in the liver (Fig. 3A). In contrast, Spink3<sup>cre</sup>; 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<sup>lacZ</sup> 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<sup>cre</sup>; 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<sup>cre</sup>; 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<sup>cre</sup>; R26R mice, but not in Spink3<sup>lacZ</sup> 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 SPIN1 protein and mRNA are expressed in mucus-producing cells of the normal gastrointestinal tract [4, 7, 13]. SPIN1 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<sup>lacZ</sup> 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<sup>lacZ</sup> mice (Figs. 4A and B). On the other hand, in adult Spink3<sup>cre</sup>; 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<sup>cre</sup>; R26R mice, Cre activity 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
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 Spink3cre; 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
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 Spink3lacZ mice (Figs. 6A and B). In Spink3cre; 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
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. Spink3cre 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. 4. β-gal activity in the gastrointestinal tract. β-gal activity was not detected in the gastrointestinal tracts of Spink3+/+;R26R (control) or Spink3lacZ mice (whole staining: A, section staining: B). However, in Spink3cre; R26R mice, β-gal activity was detected in both crypt and tip compartments of the small intestine.
Acknowledgments

We thank Mrs. Michiyo Nakata for her excellent work on tissue section preparation. This study was supported in part by Grants-in-Aid for Scientific Research (S) to K. Yamamura (21220010) from the Japan Society for the Promotion of Science, KAKENHI (24591016) to M. Ohmuraya from the Japan Society for the Promotion of Science, and a grant from the Research Committee of Intractable Pancreatic Disease (Principal investigator: Tooru Shimosegawa) provided by the Ministry of Health, Labour and Welfare of Japan.

References

1. Araki, K., Araki, M., and Yamamura, K. 2002. Site-directed integration of the cre gene mediated by Cre recombinase using a combination of mutant lox sites. *Nucleic Acids Res.* 30: e103. [Medline] [CrossRef]
2. Araki, K., Araki, M., and Yamamura, K. 2006. Negative selection with the Diphtheria toxin A fragment gene improves frequency of Cre-mediated cassette exchange in ES cells. *J. Biochem.* 140: 793–798. [Medline] [CrossRef]
3. Araki, K., Okada, Y., Araki, M., and Yamamura, K. 2010. Comparative analysis of right element mutant lox sites on recombination efficiency in embryonic stem cells. *BMC Biotechnol.* 10: 29. [Medline] [CrossRef]
4. Freeman, T.C., Playford, R.J., Quinn, C., Beardshall, K., Poulter, L., Young, J., and Calam, J. 1990. Pancreatic secretory trypsin inhibitor in gastrointestinal mucosa and gastric juice. *Gut* 31: 1318–1323. [Medline] [CrossRef]
5. Kazai, L.A., Spicer, D.S., and Brahinsky, R.A. 1948. Isolation of a crystalline trypsin inhibitor-anticoagulant protein from pancreas. *J. Am. Chem. Soc.* 70: 3034–3040. [Medline] [CrossRef]
6. Kobayashi, K., Horiuchi, M., and Saheki, T. 1997. Pancreatic secretory trypsin inhibitor as a diagnostic marker for adult-onset type II citrullinemia. *Hepatology* 25: 1160–1165. [Medline] [CrossRef]
7. Marchbank, T., Chinery, R., Hanby, A.M., Poulson, R., Elia, G., and Playford, R.J. 1996. Distribution and expression of pancreatic secretory trypsin inhibitor and its possible role in epithelial restitution. *Am. J. Pathol.* 148: 715–722. [Medline]
8. Niwa, H., Yamamura, K., and Miyazaki, J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108: 193–199. [Medline] [CrossRef]
9. Ohmachi, Y., Murata, A., Matsuura, N., Yasuda, T., Yasuda, T., Monden, M., Mori, T., Ogawa, M., and Matsubara, K. 1993. Specific expression of the pancreatic-secretory-tryp-
sin-inhibitor (PSTI) gene in hepatocellular carcinoma. Int. J. Cancer 55: 728–734. [Medline] [CrossRef]
10. Ohmuraya, M., Hirota, M., Araki, M., Mizushima, N., Matsui, M., Mizumoto, T., Haruna, K., Kume, S., Takeya, M., Ogawa, M., Araki, K., and Yamamura, K. 2005. Autophagic cell death of pancreatic acinar cells in serine protease inhibitor Kazal type 3-deficient mice. Gastroenterology 129: 696–705. [Medline]
11. Ohmuraya, M., Sugano, A., Hirota, M., Takaoka, Y., and Yamamura, K. 2012. Role of Intrapancreatic SPINK1/Spink3 Expression in the Development of Pancreatitis. Front. Physiol. 3: 126. [Medline] [CrossRef]
12. Ozaki, N., Ohmuraya, M., Hirota, M., Ida, S., Wang, J., Takamori, H., Higashiyama, S., Baba, H., and Yamamura, K. 2009. Serine protease inhibitor Kazal type 1 promotes proliferation of pancreatic cancer cells through the epidermal growth factor receptor. Mol. Cancer Res. 7: 1572–1581. [Medline] [CrossRef]
13. Playford, R.J., Batten, J.J., Freeman, T.C., Beardshall, K., Vesey, D.A., Fenn, G.C., Baron, J.H., and Calam, J. 1991. Gastric output of pancreatic secretory trypsin inhibitor is increased by misoprostol. Gut 32: 1396–1400. [Medline] [CrossRef]
14. Scheving, L.A. 1983. Primary amino acid sequence similarity between human epidermal growth factor-uрогastrone, human pancreatic secretory trypsin inhibitor, and members of porcine secretin family. Arch. Biochem. Biophys. 226: 411–413. [Medline] [CrossRef]
15. Soriano, P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 21: 70–71. [Medline] [CrossRef]
16. Stenman, U.H., Huhtala, M.L., Koistinen, R., and Seppala, M. 1982. Immunochemical demonstration of an ovarian cancer-associated urinary peptide. Int. J. Cancer 30: 53–57. [Medline] [CrossRef]
17. Wang, J., Ohmuraya, M., Hirota, M., Baba, H., Zhao, G., Takeya, M., Araki, K., and Yamamura, K. 2008. Expression pattern of serine protease inhibitor kazal type 3 (Spink3) during mouse embryonic development. Histochem. Cell Biol. 130: 387–397. [Medline] [CrossRef]
18. Witt, H., Apte, M.V., Keim, V., and Wilson, J.S. 2007. Chronic pancreatitis: challenges and advances in pathogenesis, genetics, diagnosis, and therapy. Gastroenterology 132: 1557–1573. [Medline] [CrossRef]
19. Yasuda, T., Ogawa, M., Murata, A., Ohmachi, Y., Yasuda, T., Mori, T., and Matsubara, K. 1993. Identification of the IL-6-responsive element in an acute-phase-responsive human pancreatic secretory trypsin inhibitor-encoding gene. Gene 131: 275–280. [Medline] [CrossRef]