

Gastrointestinal Hamartomatous Polyposis in *Lkb1* Heterozygous Knockout Mice<sup>1</sup>Hiroyuki Miyoshi, Masayuki Nakau, Tomo-o Ishikawa, Michael F. Seldin, Masanobu Oshima, and Makoto M. Taketo<sup>2</sup>

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## Abstract

Peutz-Jeghers syndrome (PJS) is a hereditary disorder characterized by gastrointestinal hamartomatous polyposis associated with mucocutaneous pigmentation. Germ-line mutations of the gene encoding LKB1 (STK11), a serine/threonine kinase, are identified in most PJS patients. To investigate the role of LKB1 in the PJS phenotypes, we introduced a germ-line mutation in the mouse *Lkb1* gene by homologous recombination in mouse embryonic stem cells. In most *Lkb1* (+/−) mice >20 weeks of age, hamartomatous polyps developed in the glandular stomach, often in the pyloric region. Small intestinal hamartomas also developed in approximately one-third of the *Lkb1* (+/−) mice >50 weeks of age. A genomic PCR and sequence analysis showed that all hamartomas retained both the wild-type and targeted *Lkb1* alleles, indicating that allelic loss of the wild-type *Lkb1* was not the cause of polyp formation. Moreover, the LKB1 protein level was not reduced in hamartomatous polyps compared with that in the *Lkb1* (+/−) normal gastric mucosa. In addition, the remaining allele showed neither missense mutations in the coding sequence nor produced truncated LKB1 in the hamartoma. Taken together, these data suggest that the wild-type *Lkb1* is expressed in the hamartoma at the haploid amount. Accordingly, the gastrointestinal hamartomas appear to develop because of the *Lkb1* haploinsufficiency. Although additional genetic events may be critical in hamartoma and adenocarcinoma development, these data strongly suggest that the initiation of polyposis is not the result of loss of heterozygosity in *Lkb1*.

## Introduction

PJS<sup>3</sup> is an autosomal dominant disease characterized by hamartomatous polyps of the gastrointestinal tract as well as mucocutaneous pigmentation of the lips, buccal mucosa, and digits (1, 2). Patients with PJS show an increased risk of developing cancer at relatively young ages (3, 4). Recently, *LKB1* (also termed *STK11*) on chromosome 19p13.3, which encodes a serine/threonine kinase of unknown function, was identified as a gene the mutations of which are responsible for PJS (5, 6). To date, various types of germ-line *LKB1* mutations have been identified in PJS families (reviewed in Ref. 7). In addition, LOH on chromosome 19p near *LKB1* or somatic mutations in the *LKB1* gene have been reported in hamartomas and adenocarcinomas developed in a subset of PJS patients, suggesting that *LKB1* is a potential tumor suppressor gene (8–13). Recent cell culture studies have also suggested that LKB1 functions as a tumor suppressor. Forced expression of *LKB1* in tumor cell lines results in suppres-

sion of cell growth by inducing cell cycle arrests at the G<sub>1</sub> phase (14). Phosphorylation of LKB1 by cyclic AMP-dependent kinase or by p90 ribosomal S6 kinase is essential for the suppression of cell growth (15, 16). LKB1 is also required for brhma-related gene 1-induced growth arrest (17). Moreover, LKB1 plays a key role in p53-dependent apoptosis (18). Taken together, mutations in *LKB1* may contribute to tumorigenesis through suppression of either growth arrest or apoptosis in epithelial cells. Recently, an *Lkb1* gene knockout mouse line has been reported (19). The homozygotes are embryonically lethal because of multiple developmental defects including aberrant vessel formation in the yolk sac and placenta. Expression of vascular endothelial growth factor is deregulated in the *Lkb1* homozygous tissues. It is possible that lack of LKB1 supports tumor cell growth through angiogenesis by induction of vascular endothelial growth factor. However, these studies did not investigate whether heterozygous *Lkb1* (+/−) mice develop hamartomatous tumors. Here, we describe construction of *Lkb1* gene knockout mice and demonstrate that *Lkb1* (+/−) mice develop gastrointestinal hamartomas without inactivation of the remaining wild-type *Lkb1* allele.

## Materials and Methods

**Targeting Vector Construction.** A partial cDNA fragment of *Lkb1* was amplified by RT-PCR using a primer set of *EIF* (5′-CTC GTA CGG CAA GGT GAA GGA G-3′) and *E6R* (5′-TAC AGG CCC GTG GTG ATG TTG T-3′). The amplified fragment was used as a probe for screening a 129/SvJ mouse genomic DNA library (Stratagene, La Jolla, CA). A *Bam*HI fragment including exons 1 to 7 was used for vector construction (Fig. 1A).

***Lkb1* Knockout Mice.** Mouse ES cells RW4 (Genome Systems, St. Louis, MO) were used. Homologous recombinants were screened by PCR using primers *PGKR* (5′-CTA AAG CGC ATG CTC CAG ACT-3′) and *LKB1EX* (5′-GGC GTC CCT AGA CAC ATT TCC-3′) and confirmed by genomic Southern analysis. Genotyping of the mice was performed by PCR using the primers *E6R* and *PGKR*. A *Bgl*II-*Bam*HI fragment probe was used for genotyping by Southern hybridization (Fig. 1A).

**Histological Analyses.** The methods have been described previously (20). The primary antibodies anti-αSMA monoclonal antibody (Progen Biotechnik, Heidelberg, Germany) and anti-β-catenin polyclonal antibody (Sigma Chemical Co. St. Louis, MO) were used at 50- and 500-fold dilutions, respectively.

**RT-PCR Analysis.** Total RNA was prepared from the glandular stomach using ISOGEN solution (Nippon Gene, Toyama, Japan), and cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Two primer sets were used to amplify a 585-bp fragment from exons 1 to 6 (*EIF* and *E6R*) and an 809-bp fragment from exons 1 to 8 (*EIF* and *E8R*; 5′-TGT CTG GGC TTG GTG GGA TAG G-3′), respectively.

**LOH Analysis.** Genomic DNA was extracted as described (20). Two primer sets were used to detect the targeted allele (*PGKR* and 5′-GTC ATC CAC AGC GAA AGG GTG C-3′) and the wild-type allele (5′-TAC TTC CGC CAG CTG ATT GAC G-3′ and 5′-GAG GTC GGA GAT CTT GAG TGT G-3′), respectively.

**In Situ Hybridization.** Sense and antisense RNA probes were prepared from a *Bgl*III-*Pst*I (1025-bp) fragment of the mouse *Lkb1* cDNA. Deparaffinized sections were treated with 0.2 M hydrochloric acid, followed by 5 μg/ml of proteinase K. Subsequently, they were fixed in 4% paraformaldehyde and treated with triethanolamine buffer with stepwise additions of acetic

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<sup>3</sup> The abbreviations used are: PJS, Peutz-Jeghers syndrome; JPS, juvenile polyposis syndrome; LOH, loss of heterozygosity; *Apc*, adenomatous polyposis coli (gene); αSMA, α-smooth muscle actin; ES, embryonic stem; RT-PCR, reverse transcription-PCR; TGF, transforming growth factor.

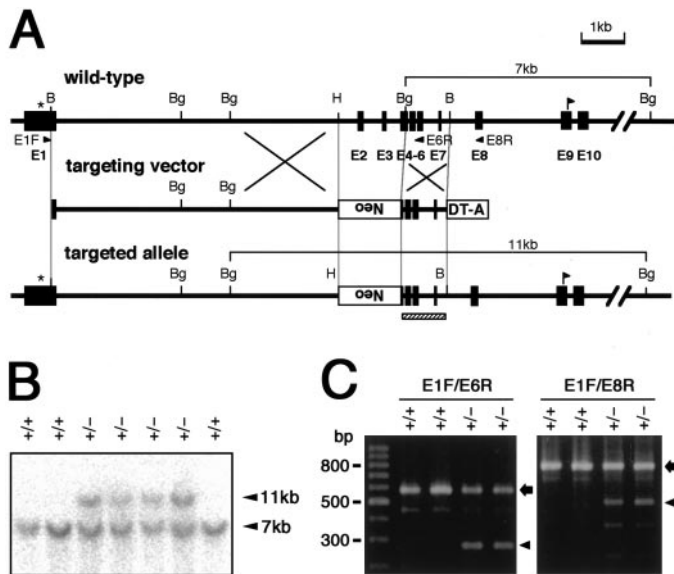


Fig. 1. Disruption of the *Lkb1* gene in mouse ES cells. **A**, schematic representation of the targeting strategy by homologous recombination. The *top part* illustrates the wild-type *Lkb1* allele, whereas the *middle and bottom parts* show the targeting vector and the targeted *Lkb1* allele, respectively. *Solid boxes*, exons; *Neo*, neomycin resistance gene cassette; *DT-A*, diphtheria toxin gene cassette; \*, initiation codons; *flags*, termination codons; *arrowheads*, PCR primer positions; *hatched box*, probe used for Southern analysis; *Bg*, *Bgl*II; *H*, *Hind*III; *B*, *Bam*HI. **B**, genotyping of the intercross offspring by Southern analysis. The 7-kb fragment corresponds to the wild-type allele, whereas the 11-kb fragment corresponds to the targeted allele. **C**, RT-PCR analysis of the wild-type and *Lkb1* (+/-) mouse gastric mucosa, amplified for exons 1 to 6 (*left*) and exons 1 to 8 (*right*). Bands for the alternatively spliced (*arrowheads*), as well as full length (*arrows*) *Lkb1* cDNA were detected in the *Lkb1* (+/-) mouse gastric mucosa.

anhydride. After prehybridization, the specimens were hybridized with digoxigenin-labeled RNA probes overnight. DIG nucleic acid detection kit (Roche) was used for signal detection.

**Western Immunoblot Analysis.** Tissue samples were homogenized and sonicated in lysis buffer [10 mM HEPES (pH 7.4), 50 mM NaCl, 50 mM sodium PP<sub>i</sub>, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 100  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 0.1% Triton X-100, and 500  $\mu$ M phenylmethylsulfonyl fluoride]. After centrifugation at 2000  $\times$  *g* at 4°C for 10 min, 20  $\mu$ g of the supernatant protein were separated in a 10% SDS-polyacrylamide gel. Sheep polyclonal anti-LKB1 antibody (Upstate Biotechnology, Lake Placid, NY) and ECL detection system (Amersham Pharmacia, Uppsala, Sweden) were used to detect the specific signals.

**Cell Isolation and Culture of Pyloric Mucosa.** The strips of mucosa from the normal and polyp tissues were prepared by peeling off the muscle layer, rinsed with the washing medium (MEM supplemented with 1% BSA, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin), and minced into <1 mm<sup>2</sup> with scissors. The minced tissue was incubated in PBS containing 1% collagenase (Invitrogen) at 37°C for 1 h with shaking. Epithelial cells were collected by centrifugation at 1000 rpm for 5 min, suspended in culture medium as described (21), and seeded on collagen-coated culture dishes to avoid contamination of interstitial cells.

**Sequencing Analyses.** cDNA fragments that covered the *Lkb1* full coding region were amplified with three sets of primers as follows: 5'-CGA AGG GGA CGA GGA CAA AGA-3' and 5'-GAA CAA TGC CCT GGC TGT GTA G-3' for the 5' part; 5'-CTG CGG CAT CGG AAT GTG A-3' and 5'-TGT CTG GGC TTG GTG GGA TAG G-3' for the middle part; and 5'-TAT GAG CCG GCC AAG AGG TTC T-3' and 5'-CTC CAA CGT CCC GAA GTG AGT G-3' for the 3' part. Amplified cDNA fragments were sequenced directly using the same primers.

## Results

**Generation of *Lkb1* Gene Knockout Mice.** The *Lkb1* gene was inactivated in ES cells by homologous recombination in which exons 2, 3, and 4 were deleted and replaced with a neomycin resistance gene cassette (Fig. 1A). The chimeric mice derived from a recombinant ES

clone transmitted the targeted *Lkb1* allele to the offspring. The progeny genotypes were examined by genomic Southern analysis (Fig. 1B). No homozygous mutant pups were obtained by intercrossing the heterozygotes, indicating that *Lkb1* (-/-) mice were embryonically lethal. This is consistent with a previous report analyzing another *Lkb1* knockout mutant (19).

The transcript from the targeted *Lkb1* allele was examined by RT-PCR analysis using primers to amplify cDNA fragments for exons 1-6 and for exons 1-8, respectively (Fig. 1A). The wild-type *Lkb1* cDNA fragment of the expected size was amplified with either primer set (Fig. 1C, *arrows*). Smaller fragments were also amplified with both primer sets only when *Lkb1* (+/-) cDNA was used as the template (Fig. 1C, *arrowheads*). The length of such shorter cDNA fragments precisely matched the predicted size of the exon 2, 3, and 4-deleted *Lkb1* cDNA. These results suggest that some alternative splicing events took place in the transcript from the targeted *Lkb1* allele. However, such alternatively spliced mRNA, in which exon 1 is ligated to exon 5, should result in a frame-shift mutation after codon 97, eliminating most of the kinase domain (residues 50-337).

**Gastrointestinal Polyps in *Lkb1* (+/-) Mice.** *Lkb1* (+/-) mice developed normally and showed no overt phenotypes up to 20 weeks of age. However, gastric polyps developed in 93% of *Lkb1* (+/-) mice >20 weeks of age (Fig. 2). After 40 weeks of age, the incidence of the gastric polyps reached 100%. Polyps developed in the glandular stomach, often in the pyloric region (Fig. 3A). Polyps were also found in the small intestine of the aged *Lkb1* (+/-) mice (Figs. 2 and 3B). The incidence of the small intestinal polyps in the heterozygotes >50 weeks was 31% (Fig. 2).

Some *Lkb1* (+/-) mice became moribund after 50 weeks of age, which was attributed to pyloric constriction and obstruction of the stomach. Others survived beyond 70 weeks of age (data not shown).

**Histological Characteristics of Gastrointestinal Polyps.** Although smaller gastric polyps were of sessile morphology, larger ones were pedunculated (Fig. 3C). They consisted of glandular and cystic epithelial layers. A bromodeoxyuridine incorporation assay showed that tumor cells proliferated continuously in the polyps (Fig. 3D). However, they did not show any dysplastic morphology, but the normal epithelial layers of different types were mixed in the polyp tissue (Fig. 3E, *inset*). These histological characteristics indicate that the *Lkb1* (+/-) polyps are hamartomas like those in human PJS. To

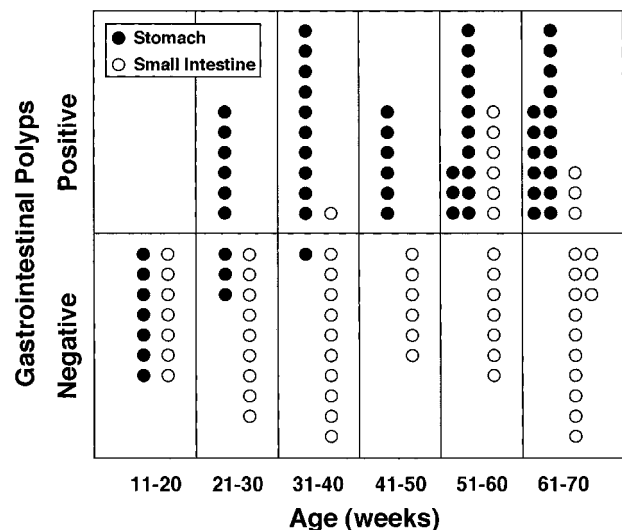


Fig. 2. Incidence of gastrointestinal polyps in the *Lkb1* (+/-) mice. Each circle represents a necropsied individual. Most mice >20 weeks of age developed gastric polyps (●), whereas the incidence of small intestinal polyps was lower (○).

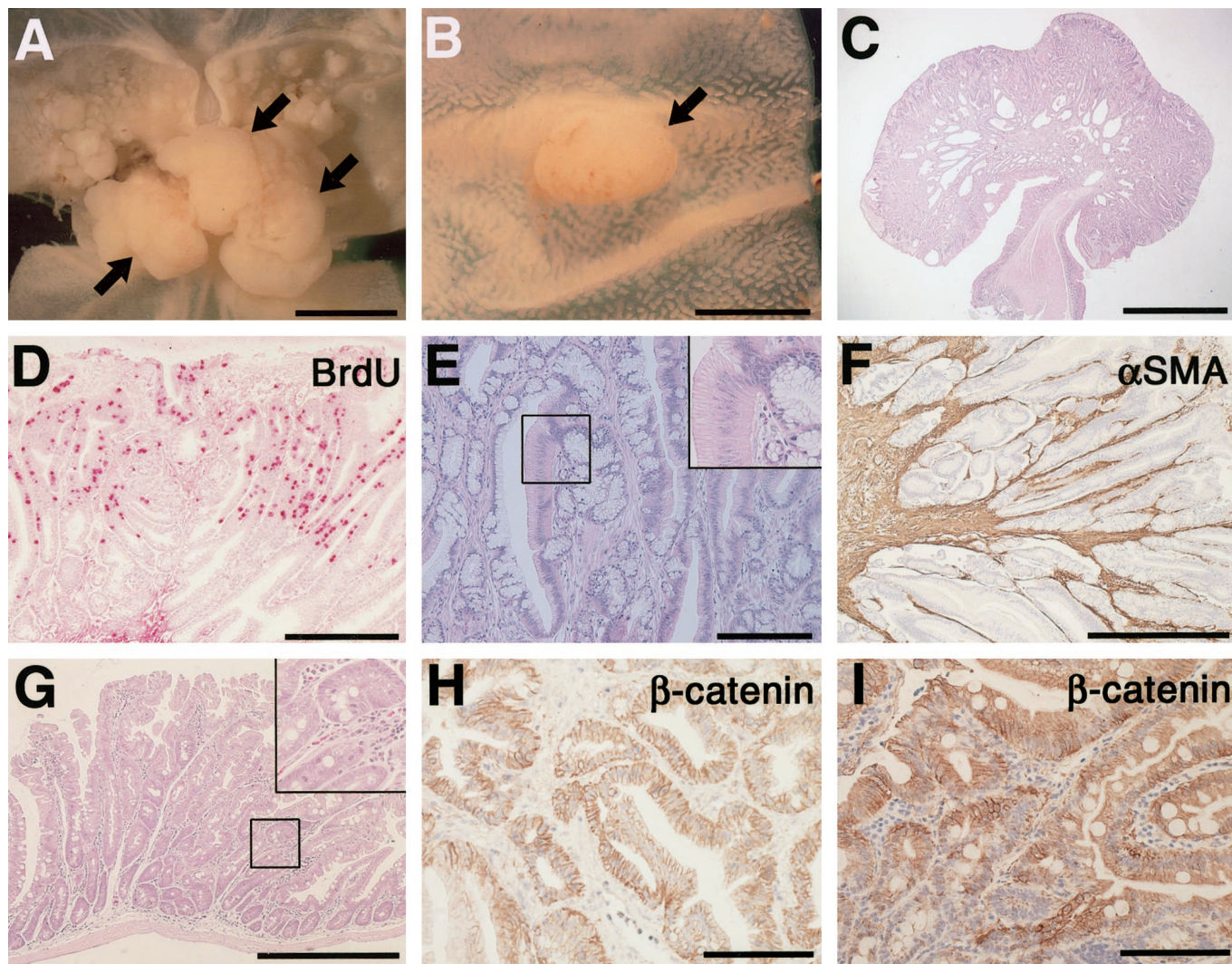


Fig. 3. Morphological and immunohistochemical examinations of gastrointestinal polyps. A and B, dissection micrographs of gastric polyps (A) and a small intestinal polyp (B). C–F, H&E staining (C and E) and immunostaining (D and F) of the gastric polyps. The large gastric polyp is pedunculated (C) and consists of different types of epithelial cells (E, inset). A bromodeoxyuridine (*BrdU*) incorporation assay shows frequent proliferation of the tumor epithelial cells (D). Immunostaining with an anti- $\alpha$ SMA antibody shows an arborizing network of smooth muscle bundles in the hamartoma (F). G, histology of a small intestinal polyp shows a similar morphology to that in the gastric polyps, H&E. H and I,  $\beta$ -catenin localizes in the basolateral membrane of a gastric polyp (H) and a small intestinal polyp (I). Bars: A, 5 mm; B and C, 2 mm; D and E, 200  $\mu$ m; F and G, 500  $\mu$ m; H and I, 100  $\mu$ m.

further compare the pathological characteristics of the *Lkb1* (+/–) with PJS polyps, we immunohistochemically studied the mouse gastric polyp sections using an anti- $\alpha$ SMA antibody. The PJS hamartomas show a particular histology where benign glands are surrounded by fronds of lamina propria containing the muscularis mucosae. In the *Lkb1* (+/–) mice, as expected, an arborizing network of smooth muscle bundles was stained with anti- $\alpha$ SMA antibody, extending into branching fronds in the polyp tissue (Fig. 3F). Thus, the *Lkb1* (+/–) polyps exhibit strikingly similar characteristics to those of PJS. The small intestinal polyps were also composed of the normal epithelial cell layers (Fig. 3G). Few adenomatous changes were observed in any hamartomas examined, which is consistent with the PJS hamartomas that show low rates of neoplastic changes (22).

These histological characteristics of the *Lkb1* (+/–) hamartomas are very different from those of the *Apc* knockout (*Apc* <sup>$\Delta$ 716</sup>) mouse intestinal polyps, where proliferating dysplastic adenoma cells are found without smooth muscle fibers of an arborizing pattern (20). To rule out the possibility that the *Wnt* signaling pathway is activated in the *Lkb1* (+/–) hamartomas, we determined the subcellular localiza-

tion of  $\beta$ -catenin by immunostaining. When the *Wnt* pathway is activated,  $\beta$ -catenin is stabilized and translocated to the nucleus, as we have demonstrated previously in the *Apc* <sup>$\Delta$ 716</sup> polyps (23). In the *Lkb1* (+/–) polyps, however,  $\beta$ -catenin was localized to the basolateral membrane and remained outside the nucleus in the polyp epithelial cells (Fig. 3, H and I). These results suggest that the *Wnt* pathway is not activated in the hamartomatous polyps, which is consistent with a previous report that neither  $\beta$ -catenin mutation nor *APC* LOH is detected in PJS hamartomas (9, 12).

**Expression of *Lkb1* in Hamartomas.** To determine whether loss of the LKB1 function caused hamartomas in the gastrointestinal epithelium, LOH for the *Lkb1* gene was analyzed in the polyp tissues. A genomic PCR analysis using allele-specific primers showed both the wild-type and targeted *Lkb1* alleles in all hamartomas examined (Fig. 4A). Then the nucleotide sequence was determined for the *Lkb1* mRNA expressed in the hamartoma epithelial cells. As a result of direct sequencing analyses, no *Lkb1* mutation was found in any of the four independent polyps examined (data not shown). Finally, LKB1 protein expression was examined by Western blotting. In *Lkb1* (+/–)

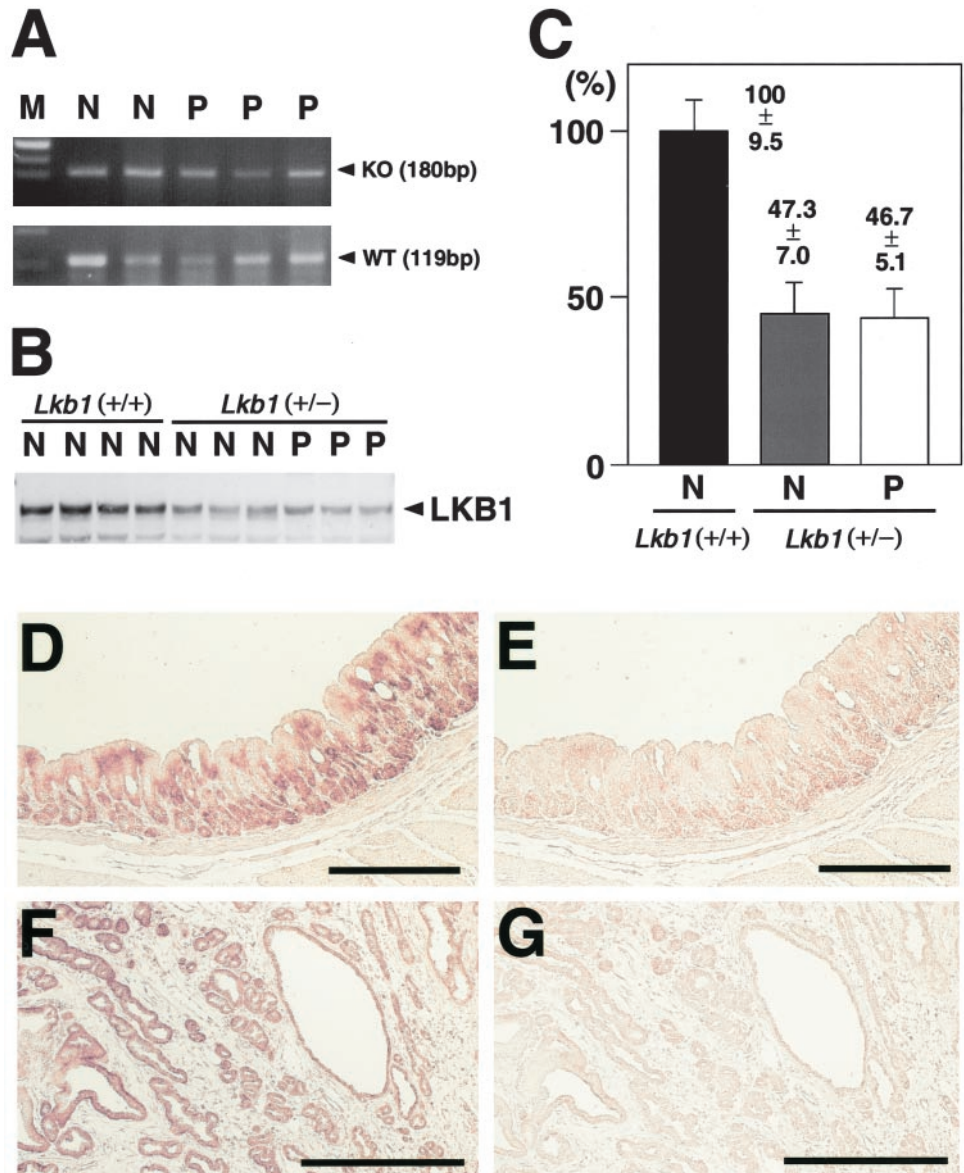


Fig. 4. *Lkb1* expression in hamartomas. **A**, a genomic PCR analysis shows that both targeted (KO) and wild-type (WT) *Lkb1* alleles are retained in the gastric hamartomas (P), as well as in the normal mucosa (N). **B**, a Western immunoblot analysis of the normal (N) and polyp (P) tissues. **C**, a histogram of quantified band intensities of (B). Compared with the wild-type mucosa, almost the half level of LKB1 is expressed in either the normal or polyp tissues of the *Lkb1* (+/-) mice. Bars, SD. **D–G**, *in situ* analysis of the *Lkb1* mRNA using an antisense probe (D and F) and a sense probe (E and G). *Lkb1* is expressed in the normal mucosa (D) and hamartoma epithelial cells (F). Bars: D and E, 200  $\mu$ m; F and G, 500  $\mu$ m.

mice, the protein expression was decreased to 47% of that in the wild-type mice, reflecting the *Lkb1* gene dosage (Fig. 4, B and C). Interestingly, the same level of LKB1 as in the normal stomach was expressed in the gastric hamartoma tissues of the *Lkb1* (+/-) mice. No shorter protein bands were detected that could be derived from the targeted allele encoding the NH<sub>2</sub>-terminal 97 residues. Taken together, these data exclude the possibility for biallelic *Lkb1* inactivation as the cause of the hamartoma initiation.

To determine the cell types that express *Lkb1* in the hamartomatous polyps, we performed an *in situ* hybridization analysis. The *Lkb1* mRNA was predominantly detected in the hamartoma epithelium, as well as in the normal epithelium of the gastric mucosa (Fig. 4, D and F). This result is consistent with a previous report that shows LKB1 expression in the polyp epithelium of PJS patients (24). These data, taken together, suggest that another genetic event(s) in addition to the heterozygous inactivation of *Lkb1* may be required for the hamartoma formation.

**Hepatocellular Carcinoma in *Lkb1* (+/-) Mice.** In extraintestinal organs, most *Lkb1* (+/-) mice developed hepatocellular carcinoma after 30 weeks of age, suggesting that LKB1 plays a tumor

suppressor role in the liver. Different from PJS, we could not find any other neoplastic lesions in *Lkb1* (+/-) mice (data not shown).

## Discussion

*LKB1* is the gene the mutations of which are associated with PJS (5, 6), and various types of germ-line *LKB1* mutations have been reported in PJS (8). The present study using *Lkb1* knockout mice provides the first direct evidence that a heterozygous *Lkb1* mutation is responsible for gastrointestinal hamartomas that share the histological features with those in human PJS. However, the polyp localization in *Lkb1* (+/-) mice is not necessarily identical with that in PJS, because polyps develop frequently in the small intestine as well as in the stomach and colon. It is conceivable that the additional genetic change that affects hamartoma formation (see below) differs between mice and human. According to some studies, LOH or somatic mutations of *LKB1* are found in informative cases of hamartomas and adenocarcinomas in PJS patients, suggesting that *LKB1* is a tumor suppressor gene (8–13). It has been also proposed that a somatic mutation in another gene may be required for the neoplastic changes of the PJS polyps (12, 13). However, it has not been determined whether loss of

the LKB1 function is essential for the hamartoma initiation. Here, we have excluded *Lkb1* LOH in the gastric hamartomas of the *Lkb1* heterozygous mice. Furthermore, we have demonstrated LKB1 expression at the haploid level in the hamartoma tissues. These data strongly suggest that biallelic inactivation of *Lkb1* is not necessarily required for the hamartoma development. It is conceivable that additional genetic alterations in other genes trigger the hamartoma formation. Considering the LKB1 functions, it is possible that LOH in the *Lkb1* gene contributes to progression of the hamartomas to malignant tumors. Because LKB1 is involved in the cell cycle arrest and p53-dependent apoptosis (14, 17, 18), reduction in its level may lead to suppression of growth arrest and apoptosis and help accumulate somatic mutations. Such cells might be predisposed to transformation. Gene chip analysis may help identify the gene mutation(s) that trigger the hamartoma formation. In this report, we have shown that the *Wnt* signaling cascade was not activated in the *Lkb1* (+/-) hamartomas. The result is consistent with a previous report that showed  $\beta$ -catenin mutation in addition to *LKB1* LOH only in adenomatous lesions of the hamartomatous polyps (9, 12). Thus, activation of the *Wnt* pathway may be an important step in hamartoma-carcinoma progression.

Previously, we demonstrated that heterozygous *Smad4* knockout mice develop gastrointestinal hamartomas that have similar histopathology to those in JPS, such as stromal expansion or association with adenomatous lesions (23). SMAD4 plays a key role in the TGF- $\beta$ -regulated signal transduction as a transcriptional factor (25), and its germ-line mutations account for a subset of familial JPS cases (26). Furthermore, heterozygous TGF- $\beta$  gene knockout mice also develop benign gastric tumors, suggesting the involvement of altered TGF- $\beta$  signaling in gastrointestinal hamartomas (27). Although histological characteristics of hamartomas in the *Lkb1* (+/-) and *Smad4* (+/-) mice are distinct as those in PJS and JPS, it is possible that similar molecular mechanisms underlie the formation of gastric hamartomas. Recently, Smith *et al.* (28) have shown that LKB1 and SMAD4 form a complex mediated by LIP1 (for LKB1 interacting protein 1). They suggest a novel mechanistic link between PJS and JPS. Therefore, it would be of great interest to investigate whether synergistic effects are observed in gastrointestinal hamartomas in *Lkb1* (+/-) *Smad4* (+/-) compound mutant mice. It is intriguing to propose that the *Lkb1* heterozygosity leads to suppression of SMAD4 transcriptional activity, resulting in hamartoma formation. It should be also important to investigate whether target genes of TGF- $\beta$  signaling is involved in hamartoma formation.

Although Knudson's two-hit model explains the majority of tumorigenesis by tumor suppressor gene inactivation, several cases have been reported recently that suggest haploinsufficiency responsible for tumor formation. For example, haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukemia in humans (29). In mice, tumors heterozygous for p53 mutation appear slightly later than those with biallelic inactivation (30). Likewise, mice hemizygous for p27<sup>Kip1</sup> develop tumors without inactivation of the remaining wild-type allele (31). It is worth noting that the homozygous mutant mice for these genes are viable and are more tumorigenic than the heterozygotes. On the other hand, homozygous mutation in *Lkb1* causes embryonic lethality. Our results indicate that haploinsufficiency may by itself predispose to tumor development and provide the first step in oncogenesis.

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