Tumorigenesis and Neoplastic Progression

Impaired Gastric Gland Differentiation in Peutz-Jeghers Syndrome

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Gastrointestinal hamartomatous polyps in the Peutz-Jeghers cancer predisposition syndrome and its mouse model (*Lkb1*^{+/-}) are presumed to contain all cell types native to the site of their occurrence. This study aimed to explore the pathogenesis of Peutz-Jeghers syndrome polyposis by characterizing cell types and differentiation of the epithelium of gastric polyps and predisposed mucosa. Both antral and fundic polyps were characterized by a deficit of pepsinogen C-expressing differentiated gland cells (antral gland, mucopeptic, and chief cells); in large fundic polyps, parietal cells were also absent. Gland cell loss was associated with an increase in precursor neck cells, an expansion of the proliferative zone, and an increase in smooth muscle α -actin expressing myofibroblasts in the polyp stroma. Lack of pepsinogen C-positive gland cells identified incipient polyps, and even the unaffected mucosa of young predisposed mice displayed an increase in pepsinogen C negative glands (25%; $P = 0045$). In addition, in small intestinal polyps, gland cell differentiation was defective, with the absence of Paneth cells. There were no signs of metaplastic differentiation in any of the tissues studied, and both the gastric and small intestinal defects were seen in *Lkb1*^{+/-} mice, as well as polyps from patients with Peutz-Jeghers syndrome. These results identify impaired epithelial differentiation as the earliest pathological sign likely to contribute to tumorigenesis in individuals with inherited *Lkb1* mutations. (*Am J Pathol* 2010, 176:2467–2476; DOI: 10.2353/ajpath.2010.090519)

mucocutaneous pigmentations, and a predisposition to cancer development.^{1–3} Peutz-Jeghers polyps have a properly polarized and apparently well-differentiated epithelium⁴ native to the site of occurrence.^{5,6} Although both polyps and carcinomas commonly arise in the gastrointestinal tract of PJS patients, the relationship between the two remains unclear. Inactivating mutations in the gene encoding for the LKB1 kinase cause PJS,¹ and mice heterozygous for *Lkb1* model the disease by developing multiple characteristic Peutz-Jeghers polyps, particularly in their gastric mucosa.⁷ The mechanism by which *Lkb1* deficiency leads to polyps appears to involve the underlying stroma, based on polyps arising even when *Lkb1* mutations are limited to the stromal smooth muscle lineage.⁸

The normal gastric mucosa consists of parallel bipartite units of basal glands and luminal pits. The appropriate cell type for each position in the unit is thought to depend on gradients of morphogens like Wnt, Hedgehog, bone morphogenetic protein, and transforming growth factor- β ,^{9–11} whose characteristic expression patterns are disrupted in gastric cancer or by inflammatory stimuli.^{12–14} The gastric gland is subdivided into isthmus, neck, and base regions. The isthmus contains stem cells,¹⁵ and a variety of precursor cells,¹⁶ which migrate bidirectionally out of the isthmus during differentiation. Throughout the stomach, pre-pit precursor cells move upwards to become Muc5a-secreting pit (foveolar) cells. Downward from the isthmus, cells migrate to form glands, specialized according to location. In the proximal glandular stomach, the glands are fundic (oxyntic), whereas pyloric antral glands cover the distal stomach. Acid-producing parietal cells^{17,18} are only found in fundic

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Peutz-Jeghers syndrome (PJS), presents as a triad of characteristic polyps with branching smooth muscle core,

glands, whereas enteroendocrine cells producing gastrin are unique to antral glands. The similarities in migration and differentiation of the fundic zymogenic chief cells and antral pyloric gland cells are considerable. Fundic pre-neck cell precursors (corresponding to antral pre-gland cells) differentiate into mucin producing neck/mucous neck cells in the fundus (differentiating gland cells in the antrum),¹⁶ and further into mucopeptic (prezymogenic) cells in the fundus (pyloric gland cells in the antrum)¹⁶ as they migrate down. The mature, differentiated pyloric gland cells express pepsinogen C,^{19,20} mucin 6 (Muc6) and trefoil factor 2 (TFF2),²¹ that also mark early mucopeptic cells. The latter gradually transform from mostly mucin-producing cells into cells mostly producing proteolytic enzymes, reaching the base of the fundic gland as mature chief cells, no longer expressing mucin.¹⁶ In mouse, chief cells are also the major producers of intrinsic factor (IF),²² whereas human IF is expressed mainly, although not exclusively, in parietal cells.²³ The signals mediating gland cell migration and differentiation in the distal stomach are only partially understood, but the transition from mucopeptic cells to chief cells requires the activity of transcription factor Mist1²⁴ and can be inhibited by stimulation of activin II receptor.²⁵ The development and persistence of pepsinogen-expressing cells, and particularly chief cells, require glucocorticoids,^{26,27} whose homeostatic effects are mediated via the mesenchymal stroma.²⁸

This stroma, the lamina propria, consisting of fibroblasts, endothelial, and immune cells, is located between and underneath the gastric units. The unit bases, the glands, are surrounded by a sheath of smooth muscle α -actin (SMA)-expressing intestinal subepithelial myofibroblasts,²⁹ which, at least in the small intestine, link with the thin muscular sheath under the basal lamina called muscularis mucosae.³⁰ In some pathological conditions of the gastric epithelium, like neo- or metaplasia^{31,32} or helicobacter gastritis,³³ these myofibroblasts increase. In polyps of Peutz-Jeghers patients³⁴ and *Lkb1*^{+/-} mice,⁷ thick disorganized bundles of smooth muscle originating from the muscularis mucosae branch like a cauliflower stalk into polyp lobes. Here we studied the epithelia of gastric polyps and predisposed stomach in *Lkb1*^{+/-} mice and Peutz-Jeghers patients to explore pathogenesis of Peutz-Jeghers polyps.

Materials and Methods

Animal and Clinical Specimens

Lkb1^{+/-}³⁵ and wild-type control mice in F1 and F2 C57BL6; CD1 backgrounds were sacrificed at an age of 4.5 months, or 10 to 11.5 months. Tissue specimens were collected and fixed in 4% paraformaldehyde for paraffin embedding as previously described.³⁶ All animal experimentation was performed with permission from and in accordance with the recommendations of the local animal welfare committee at the University of Helsinki and State Provincial Office of Southern Finland. Paraffin-embedded samples of polyps and biopsies of normal gastric tissue from Peutz-Jeghers patients were obtained from the archives of the Helsinki University Central Hos-

pital Pathology Department, in accordance with local ethical regulations.

Immunohistochemistry

All immunohistochemistry was performed on dewaxed and rehydrated sections, with endogenous peroxidase quenched by 3% hydrogen peroxide in either PBS or methanol. For staining against H⁺/K⁺-ATPase (HA3-923, Affinity Bioreagent, Golden, CO), Cdx2 (MU392A-U, BioGenex Laboratories, San Ramon, CA), chromogranin A (A0430, DakoCytomation, Carpinteria, CA), Muc6 (NCL-MUC-6, Novocastra, Newcastle On Tyne, UK), or Ki-67 (Clone TEC-3, DakoCytomation), sections were pretreated with DAKO Target Retrieval (DakoCytomation) at 95°C, 20 minutes. H⁺/K⁺-ATPase signal was detected with the ARK-kit (DakoCytomation), whereas Ki-67 and CgA signal with biotinylated anti-rat- and anti-rabbit-antibodies (Vector Laboratories, Burlingame, CA), ABC-reagent (Vector) and diaminobenzidine substrate (Sigma-Aldrich, St. Louis, MO). Muc6 signal was detected with AlexaFluor488 conjugated anti-mouse antibody (Invitrogen, Carlsbad, CA). TFF2 (a kind gift by Dr. George Elia, Histopathology Unit, ICRF, London) and gastrin (A0568, DakoCytomation) antibodies were used without pretreatment, and detected with fluorescein isothiocyanate-conjugated anti-mouse IgM (Vector) and with biotinylated anti-rabbit-antibody (Vector), and ABC-reagent with 3-amino-9-ethylcarbazole substrate (Vector) respectively. For staining with alkaline phosphatase-conjugated α -SMA (A5691, Sigma-Aldrich), sections were incubated at 37°C, 30 minutes in 0.02% Trypsin (Difco Laboratories, Detroit, MI) in 0.05 mol/L Tris-HCl-CaCl₂ (pH 7.6) and unspecific antibody binding blocked with blocking reagent in the TSA-kit (Perkin Elmer, Foster City, CA). Signal was detected by addition of Black Alkaline Phosphatase Substrate (Vector). Desmin antibody (DE-R-11, Novocastra, Newcastle, UK) and lysozyme antibody (A0099, DakoCytomation) were used after similar pretreatment as α -SMA. Desmin was detected with the ARK-kit, lysozyme and biotinylated *Griffonia simplicifolia* (GS) lectin II (B-1215, Vector) were detected with ABC-reagent and 3-amino-9-ethylcarbazole substrate. Staining against pepsinogen C was performed with an antibody against rat Pg-1^{37,38} kindly provided by Dr. T. Tsukamoto (Aichi Cancer Center Research Institute, Nagoya, Japan) and staining against IF with a polyclonal rabbit antibody kindly contributed by Dr. D.H. Alpers (Washington University School of Medicine, St. Louis, MO) according to the respective recommended protocols. All immunostainings except TFF2, IF, and Ki-67 were counterstained with hematoxylin (Shandon Instant Hematoxylin, Thermo Fisher Scientific, Waltham, MA). Foveolar cells were detected with Alcian blue-Periodic acid-Schiff (AB-PAS) staining.

Quantification and Statistical Analysis

The quantified tissue sections were derived from the proximity of the major curvature. Pepsinogen altered py-

loric glands (PAPG) were determined in 4.5-month-old mice by blinded scoring of 115 ± 30 pyloric glands (= bases of antral gastric units) per stained section as either positive or negative for pepsinogen C. The pylorus itself was excluded from the score, and subsequent analysis, as were weakly positive glands ($2.8 \pm 1.8\%$ of all counted glands in wild-type and $3.4 \pm 1.7\%$ in $Lkb1^{+/-}$ mice). Sizes of TFF2 expressing cells were manually scored as the cross section area in micrographs of stained tissue sections, using ImageJ software (<http://rsb.info.nih.gov/ij/>). Cell numbers in the fundic stomach of aged (10- to 11.5-month-old mice) were scored from gastric units assessable in their full length. The units were selected 5 to 10 mm from the border to the squamous forestomach (the fundic-most part of murine stomach). The significance of the differences between groups was determined with Mann-Whitney *U*-tests, using the SPSS-software package (SPSS Inc., Chicago, IL). All values are given as mean $\pm$ SD.

GeneChip Arrays and Analysis of Gene Expression Data

Tissue samples were prepared from the fundus of 8-month-old littermate mice. Total RNA samples were collected using the RNEasy-kit (Qiagen, Valencia, CA) from pieces of wild-type stomach wall ($n = 6$), $Lkb1^{+/-}$ stomach wall ($n = 6$) approximately 5 mm in diameter, and from $Lkb1^{+/-}$ fundic polyps ($n = 4$). Amplification and labeling of mRNA were performed with Superscript II (Qiagen, Valencia, CA) and ENZO (Enzo Life Sciences, Farmingdale, NY) kits, according to the manufacturers' protocols. Expression profiling was conducted with Mg-U74a2 GeneChips (Affymetrix, Palo Alto, CA), also according to the manufacturer's instructions. Expression profiles were normalized using the GC-RMA method within the GeneSpring GX software (Agilent Technologies, Santa Clara, CA). Expression of genes reported to be enriched in foveolar cells,³⁹ chief cells,⁴⁰ and parietal cells⁴¹ were compared between wild-type and $Lkb1^{+/-}$ tissues. Identifiers of the published gene lists were translated to Mg-U74a2 probe set identifiers using the NetAffx batch query tool (Affymetrix, Santa Clara, CA). In addition, a random control gene list was generated from all

Figure 1. Abnormally proportioned gastric units and lack of terminal gland cell differentiation in large gastric $Lkb1^{+/-}$ polyps with characteristic Peutz-Jeghers features. **A:** Comparison of proliferating (Ki-67), foveolar (Alcian blue-Periodic acid-Schiff; AB-PAS) and glandular cells (*Griffonia simplicifolia*-lectin; GS-lectin) in antral mucosa of wild-type and $Lkb1^{+/-}$ mice, and in an antral polyp demonstrating a widening of the proliferating, foveolar (f), and glandular (g) zones in an antral $Lkb1^{+/-}$ polyp, at $\times 200$ magnification. Scale bars = 100 μ m. **B:** Staining of an antral polyp with desmin, GS-lectin, and AB-PAS demonstrates that GS-lectin positive gland-type cells are more abundant in the vicinity of desmin staining smooth muscle bundles (arrowheads). Micrographs recorded at $\times 100$ magnification. Size bars = 200 μ m. **C:** A fundic polyp demonstrating lack of pepsinogen C expressing zymogenic/chief cells and parietal cells (Hydrogen-potassium adenosine-triphosphatase; H^{+}/K^{+} -ATPase), whereas GS-lectin positive mucous neck cells (n) and foveolar cells (AB-PAS, arrowhead) are abundant. **Inset** in GS-lectin panel demonstrates that GS-lectin positive mucous neck cells (n) cover the gland base adjacent to the smooth muscle stalk (m), whereas in normal glands the base contains chief cells (c). The surface of this polyp had suffered an erosion (asterisks).

genes containing the letters G and A in their GeneSpring GX annotation (196 identifiers). For each list, the average and standard deviations of the expression fold change were calculated, and a two-way analysis of variance test for significant differences in the expression levels between the experimental groups was performed using the R statistical analysis environment. Posthoc analysis was done with Student's *t*-test.

Results

Lack of Terminal Glandular Differentiation in *Lkb1*^{+/-} Polyps

Analysis of Peutz-Jeghers type gastric polyps from 10- to 11.5-month *Lkb1*^{+/-} mice revealed a widened proliferative zone as recognized by Ki-67 staining (Figure 1A). Foveolar cell differentiation upwards from the proliferating zone appeared normal based on AB-PAS staining demonstrating neutral mucus-containing cells at the surface of both unaffected normal mucosa and in polyps. Also initial gland cell differentiation appeared normal based on staining with the GS lectin II (GS-lectin) in antral (Figure 1A) and fundic (Figure 1C) mucosa. This was also observed when staining polyps with an antibody against

Muc6 (Supplemental Figure S1 at <http://ajp.amjpathol.org>). While the proportion of foveolar cells to the GS-lectin positive cells in unaffected *Lkb1*^{+/-} mucosa was comparable with that of controls, it varied considerably within and among polyps. In the proximity of smooth muscle bundles, the epithelium contained a higher proportion of GS-lectin positive cells, while elsewhere the epithelium contained more foveolar cells (Figure 1B).

Surprisingly, both antral and fundic polyps demonstrated either weak (*n* = 26/47) or no (*n* = 21/47) pepsinogen C staining, indicating a deficiency in antral gland, mucopeptic, and chief cells not observed in the unaffected mucosa; a fundic polyp is shown in Figure 1C. Large fundic polyps also demonstrated absence of parietal cells based on morphological analysis and H⁺/K⁺-ATPase-antibody staining (Figure 1C). Concomitantly with lack of terminally differentiated gland cells, their precursors reached the gland base, based on GS-lectin staining (Figure 1C, inset).

Absence of Pepsinogen C Identifies Incipient Peutz-Jeghers Polyps

Apart from large polyps with typical Peutz-Jeghers features, the antral mucosa of *Lkb1*^{+/-} mice contains areas

Figure 2. Aberrant epithelium and stroma in incipient *Lkb1*^{+/-} polyps. **A:** Staining of proliferating (Ki-67) and pyloric gland cells (pepsinogen C) in antral mucosa of wild-type and *Lkb1*^{+/-} mice, and in an incipient polyp (bracket). All micrographs at ×100 magnification. Scale bars = 200 μm. The normal gastric folds of the section (*) at the proliferative zone (Ki-67) without disrupting the pepsinogen C staining pattern (arrowhead). **B:** Decreased pepsinogen C expression in an incipient antral *Lkb1*^{+/-} polyp (bracket) is associated with accumulation of neck cells (GS-lectin, arrow), and an increase in smooth muscle α-actin (SMA) expression (arrowheads; ×100 magnification. Scale bar = 200 μm. **C:** Pepsinogen C staining of antral mucosa in 4.5-month-old *Lkb1*^{+/-} mice reveals an increase in pepsinogen C deficient glands (pepsinogen altered pyloric glands, PAPG, marked with asterisks in micrograph) compared with the wild-type. The frequencies of PAPG (PAPG %) are shown in the bar chart, *n* = number of glands (number of mice in parentheses). **D:** Triple staining with SMA (black chromogen), pepsinogen C (red chromogen), and Ki-67 (brown chromogen) of antral mucosa from a 4.5-month-old mouse demonstrates pepsinogen C deficient glands (left panel, asterisks; ×200 magnification) and Ki-67 positive proliferating cells (arrowhead) at the base of a pepsinogen C negative gland in the right panel (×630 magnification).

with varying features of hyperplasia or hyperplastic polyposis characterized as incipient polyps.³⁶ Also in these, a deficiency in pepsinogen C staining was observed, together with a widened proliferative zone (Figure 2A, incipient polyp). Indeed, reduced pepsinogen C staining identified small incipient polyps from gastric folds, sometimes similar in appearance in tissue sections (Figure 2A, wild-type). The interglandular stroma of the incipient polyps showed increased SMA, compared with surrounding mucosa (seen in 42/72 polyps with a 0.2 to

2.0 mm diameter) associated with an increase in GS-lectin positive epithelial cells (Figure 2B) demonstrating a correlation between stromal and epithelial alterations already in incipient Peutz-Jeghers polyps.

Pepsinogen-Deficient Glands Precede Macroscopic Polyposis

A small fraction (ca. 6%) of normal gastric pyloric glands have been found to contain strongly reduced or undetectable pepsinogen C,³⁷ and these PAPGs increase following carcinogen exposure.^{37,38} To investigate possible alterations in the frequency of these altered glands in *Lkb1*^{+/-} mice before polyposis, antral mucosa of young (4.5 mo) mice without detectable hyperplasia or polyps was analyzed for PAPGs (Figure 2C). The frequency was significantly increased in *Lkb1*^{+/-} glands, compared with wild-type littermates ($8.6 \pm 2.5\%$ vs. $6.4 \pm 2.3\%$; $P = 0.045$). Furthermore, concomitant staining with Ki-67 demonstrated that some pepsinogen C negative glands contained proliferative cells at the gland base (Figure 2D, arrowheads), and the frequency was significantly higher in *Lkb1*^{+/-} mice 19/47 (40%) compared with controls (2/13; 15%). Excluding these focal alterations, proliferation in the unaffected antral mucosa in these young mice was not altered between *Lkb1*^{+/-} (11.0 ± 2.5 Ki-67 positive cells per gland) and wild-type (11.2 ± 2.22 cells) mice.

These results indicate that the unaffected but predisposed *Lkb1*^{+/-} antral epithelium in young mice contains differentiation defects similar to those detected in polyps of older mice. Interestingly, the lack of pepsinogen C and any expansion of Ki-67 were not associated with any detectable increase in SMA-positive stromal cells (Figure 2D), suggesting that the epithelial defects seen in the young mice would precede stromal defects observed in incipient and large polyps. On the other hand, in mice with a conditional ablation of *Lkb1* only in stromal *Tagln*-CRE positive cells (*Lkb1*^{lox/+}; *Tagln*^{Cre/+} mice⁸) pepsinogen C staining was undetectable in four large polyps, weak in two other large polyps (Supplemental Figure S2 at <http://ajp.amjpathol.org>) and similar to surrounding epithelium in two small polyps, demonstrating that *Lkb1* defects limited

Figure 3. Alterations in the expression of cell-type specific gene sets in *Lkb1*^{+/-} tissues, and the exclusion of spasmodic polypeptide-expressing metaplasia in *Lkb1*^{+/-} fundic polyps. **A:** Average fold change $\pm$ SD of gene expression in indicated cell-type specific gene sets including a random set for comparison (please see *Materials and Methods*) in *Lkb1*^{+/-} polyps (black) and unaffected fundic mucosa (gray) compared with wild-type mucosa. *P* values indicate the statistical significance of the difference between the wild-type and the corresponding value, as given by the Student's *t*-test. **B:** TFF2 and IF staining of *Lkb1*^{+/-} fundic polyps and wild-type mucosa, demonstrating absence of IF in the polyp (upper row) at $\times 100$, and a magnification of the area within the white box in the upper right panel. Scale bars = 200 μ m. In the lower panels, micrographs at $\times 630$ magnification (Scale bars = 32 μ m) show fundic *Lkb1*^{+/-} mucosa (left panel) and only TFF2-staining gland cells in the polyp proper (middle panel) as well as a gland with a single IF-positive cell from the border of a polyp base (right panel). **C:** Gastrin and chromogranin A (CgA) staining of *Lkb1*^{+/-} antrum, fundus and a fundic polyp provide no evidence for antralization (antrum and fundus at $\times 200$ magnification; polyp at $\times 100$ magnification; Scale bars = 100 μ m).

to stroma affect gland cell differentiation as the *Lkb1*^{lox/+}; *Tagln*^{Cre/+} polyps mature.

Altered Epithelial Composition Reflected in Gene Expression Profiles

To further investigate the epithelial differentiation defects in *Lkb1*^{+/-} polyps, we searched the literature for gene expression sets characterizing different gastric cell types, and compared wild-type, and *Lkb1*^{+/-} fundic mucosa, and fundic *Lkb1*^{+/-} polyps for the expression of genes in such sets described for foveolar cells,³⁹ chief cells⁴⁰ and parietal cells.⁴¹ The combined analysis of expression signatures in the three cell type sets by two-way analysis of variance demonstrated significant alterations in the *Lkb1*^{+/-} polyps ($P < 2.2 \times 10^{-16}$), corroborating the epithelial composition changes seen in the immunohistochemical analysis.

Closer inspection of the expression profiles of *Lkb1*^{+/-} polyps revealed that the expression level was below that of the wild-type mucosa in 73.0%(65/89) of the gene identifiers in the chief cell set and in 81.7%(98/120) of the parietal cell set. For the foveolar cell set, expression in the polyps was above that of the wild-type mucosa in 70.6%(303/429). Unaffected *Lkb1*^{+/-} mucosa, on the other hand, was unaltered with regard to the expression of these gene sets. These results, are depicted in Figure 3A, as averages for each cell type gene set expression in polyps (black) and unaffected *Lkb1*^{+/-} fundic mucosa (gray), given as fold change from the expression in wild-type mucosa. *P* values indicate where the averaged expression differs significantly from the wild-type expression level (normalized to 1.0 ± 0.0). Also expression of an independent set of 54 chief cell gene identifiers³⁹ not shown in the figure, was significantly ($P = 0.0018$) decreased in fundic polyps (average expression fold change 0.88 ± 0.36) whereas unaffected *Lkb1*^{+/-} fundic mucosa had a similar expression signature for this set as the wild-type (1.03 ± 0.16).

Despite the similarity between wild-type and unaffected *Lkb1*^{+/-} mucosa observed in the above gene expression array experiments, manual counting of marker-labeled cells in immunostained tissue sections, pointed to a small increase in foveolar cells and decrease in chief cells and mucopeptic cells (Supplemental figure S3 at <http://ajp.amjpathol.org>), not fully excluding minor alterations of epithelial composition also in the unaffected *Lkb1*^{+/-} mucosa.

Fundic Polyp Glands Distinct from Spasmolytic Polypeptide Expressing Metaplasia

In the gastric fundus, antralization and spasmolytic polypeptide (TFF2) expressing metaplasia are known as a route to loss of glandular epithelial differentiation.⁴² This led us to further examine fundic *Lkb1*^{+/-} polyps for features of antralization. Large gland cells expressing both TFF2 and IF in different subcellular compartments are typical for spasmolytic polypeptide-expressing metapla-

sia.⁴³ Here, in fundic polyps, TFF2 expression levels were comparable with those normally found in fundic neck cells and overlapping with the pattern of GS-lectin/Muc6 staining. The size of TFF2 expressing cells was the same

Figure 4. Impaired gland cell differentiation in human gastric Peutz-Jeghers polyps. **A:** Pepsinogen C or GS-lectin staining of sections from the base of an antral Peutz-Jeghers patient polyp or control gastric mucosa from a patient biopsy specimen (all micrographs at $\times 100$ magnification; Scale bar = 200 μ m). **B:** Staining for H⁺/K⁺-ATPase demonstrates deficiency in parietal cells in the polyp and staining for chromogranin A (CgA) shows presence of enteroendocrine cells in the base of a fundic Peutz-Jeghers patient polyp (at $\times 100$ magnification; Scale bar = 200 μ m). **C:** Comparison of gastrin positive cells in antral and fundic polyp indicates presence according to the native site of occurrence (also at $\times 100$ magnification; Scale bar = 200 μ m).

Figure 5. Correct specification into gastric versus intestinal epithelium in Peutz-Jeghers polyps and impaired Paneth cell differentiation in intestinal polyps. **A:** AB-PAS and TFF2 stainings of *Lkb1*^{+/-} polyps from jejunum and antrum, indicating intestinal (acid) type mucin in cells with goblet morphology in the jejunal polyp consistent with normal jejunal staining pattern, and gastric (neutral) mucin on the surface of the antral polyp, as in normal gastric mucosa (micrograph at $\times 400$; Scale bar = $50\ \mu\text{m}$). TFF2 is expressed in an antral polyp, and in the jejunal polyp there is no ectopic expression (**middle** panels, overlaid to the right with guides highlighting the basal surface of the epithelium in magenta, and the apical surface in blue), at $\times 200$ magnification; Scale bar = $100\ \mu\text{m}$. **B:** Staining of intestinal transcription factor Cdx2 in fundic and small intestinal *Lkb1*^{+/-} mucosa and polyps indicates the Cdx2 expression level in the polyps is comparable that of the native mucosa. However, Cdx2 expression extends to the crypt bottoms in the polyps (panel "Intestinal polyp base") unlike in normal mucosa. All micrographs at $\times 200$ magnification; Scale bar = $100\ \mu\text{m}$. **C:** Staining for the Paneth cell marker lysozyme shows absence in polyps both from *Lkb1*^{+/-} mice (**right-hand inset**) and PJS patients (**bottom right panel**) compared with unaffected small intestine (**left-hand inset** and **bottom left panel** respectively). Mouse intestinal cross section at $\times 50$ magnification (**insets** at $\times 200$), patient polyp and control tissues at $\times 100$ magnification (Scale bars = $200\ \mu\text{m}$).

in polyps and in normal fundic epithelium ($78.2 \pm 33.1\ \mu\text{m}^2$ in two fundic polyps from two *Lkb1*^{+/-} mice vs. $78.7 \pm 26.2\ \mu\text{m}^2$ in a wild-type mouse). We also found that IF was absent from the fundic polyps similarly to pepsinogen C (Figure 3B, upper panels and lower middle panel). At the edges of their bases, 2/6 fundic polyps contained a couple of astray IF-expressing cells, which, however, had no concomitant TFF2-expression (Figure 3B lower rightmost pane). Also, staining for gastrin did not reveal ectopic expression in either fundic polyps or normal fundus of *Lkb1*^{+/-} mice, whereas gastrin-producing cells were present in antral mucosa (Figure 3C), providing no further evidence for antralization in fundic *Lkb1*^{+/-} polyps.

Impaired Gland Differentiation in Human Gastric Peutz-Jeghers Polyps

The defective gland cell differentiation in gastric polyps of *Lkb1*^{+/-} mice next prompted us to study PJS patient polyps for similar defects. Pepsinogen C staining of five fundic and two antral PJS patient polyps was undetectable (4/7) or weak (3/7) compared with that of unaffected fundic mucosa (Figure 4A). Also the PJS patient fundic polyps contained no detectable parietal cells (Figure 4B). Chromogranin A positive cells were seen in all polyps at normal frequencies, and gastrin expression was found to be limited to antral polyps (Figure 4C), signifying that these enteroendocrine cells were present only when na-

tive to the site of the occurrence of the polyp. These results demonstrated that in PJS patient polyps, epithelial differentiation is impaired similarly as in *Lkb1*^{+/-} mice.

Lysozyme Deficiency in Intestinal Peutz-Jeghers Polyps

While the focus of this study was on the characterization of the abundant gastric polyps, we included three jejunal mouse polyps and four duodenal PJS patient polyps to address possible misspecification along the horizontal axis of gastrointestinal differentiation, reported in juvenile polyposis.⁴⁴ AB-PAS staining suggested the mucins to be correctly specified horizontally, and no detectable staining of the stomach specific TFF2 was seen in the jejunal polyps (Figure 5A). Also, while in the intestinal polyps expression of intestinal transcription factor Cdx2 was robust, no staining was detected in the gastric polyps (Figure 5B) arguing against intestinal metaplastic transformation.⁴⁵ Interestingly however, Cdx2 was present also in the very basal cells of the intestinal polyp crypts (Figure 5B, intestinal polyp base), where it normally would be down-regulated to allow for Paneth cell differentiation.⁴⁶ Consistent with this, direct analysis of Paneth cell differentiation using lysozyme staining demonstrated decreased or absent expression in both mouse and human polyps (Figure 5C). This demonstrated a gland cell differentiation defect also in small intestinal Peutz-Jeghers polyps.

Discussion

This investigation revealed severe disruption of gland cell differentiation in gastric Peutz-Jeghers polyps through the immunohistochemical study of gastric cell type markers as summarized in a data table (Supplemental table T1, at <http://ajp.amjpathol.org>), and a schematic of a gastric unit within a Peutz-Jeghers polyp, compared with normal mouse antral and fundic glands (Figure 6). Fundic glands are found in the anatomical fundus of the mouse stomach, and in the anatomical fundus and corpus of the human stomach (lower schematic, Figure 6). The disruption of gland cell differentiation was also supported by alterations in the gene expression profile of *Lkb1*^{+/-} polyps, which indicated a reduction in mRNAs characterizing parietal and chief cells, and an increase in mRNAs linked to mucus producing foveolar cells.

The most notable change observed in the *Lkb1*^{+/-} gastric epithelium was a deficiency in terminally differentiated peptic gland cells, demonstrated both in antral and fundic polyps by a lack of pepsinogen C staining. Furthermore, the predisposed mucosa of young, polyp-free mice displayed a significant increase in glands lacking terminally differentiated pyloric gland cells. A similar increase in pepsinogen altered pyloric glands has been described as the first detectable change on carcinogen-induction of gastric cancer, and shown to associate with increased proliferation.³⁷ In this study, we further showed that proliferative cells replaced the pepsinogen C expressing cells at the bases of the altered glands, suggesting disrupted homeostasis. As these changes preceded the formation of polyps and of any noticeable stromal alterations, they represent the first observed defect due to *Lkb1* heterozygosity, and should thus be considered a possible causative event for Peutz-Jeghers polyp formation.

On the other hand, several observations suggest that the epithelial changes detected are secondary to stromal alterations. Conditional deletion of *Lkb1* in stromal smooth muscle cells (*Lkb1*^{lox/+}; *Tagln*^{Cre/+}) was recently shown to cause a polyposis very similar to that observed in *Lkb1*^{+/-} mice.⁸ Also, in this study we observed an increase in SMA-positive stromal cells in incipient polyps (Figure 2). This is consistent with a model, wherein epithelial differentiation defects would be secondary to altered stromal-epithelial signaling necessary for peptic cell differentiation.^{28,47} Here, similarly disrupted gland cell differentiation in polyps from *Lkb1*^{+/-} mice and from *Lkb1*^{lox/+}; *Tagln*^{Cre/+} mice supported such a model. Although *Lkb1*^{lox/+}; *Tagln*^{Cre/+} polyps thus appear indistinguishable from *Lkb1*^{+/-} polyps also in this regard, they are smaller and occur in only 61% of mice at 11 months, compared with 100% in the *Lkb1*^{+/-} mice.⁸ Based on these observations, it is thus possible that in *Lkb1*^{+/-} mice, here undetected stromal alterations precede epithelial differentiation defects, which in turn are even further enhanced by concomitant epithelial *Lkb1* heterozygosity, contributing to increased polyp initiation and growth.

Considering the order in which the differentiation defects may occur in the *Lkb1*^{+/-} gastric epithelium one

Figure 6. Schematic comparing a gastric unit in a Peutz-Jeghers polyp to fundic and antral ones. The PJS polyp gastric unit is even more elongated, with irregular morphology. (Note that normal human gastric units are larger and may also be more irregular than their mouse counterparts; several antral glands may, for example, empty into one pit.) Numerous proliferating cells in the unit neck feed into a pit region of foveolar cells and a basal region of gland-type cells (cells in transit both proliferate and contain differentiation markers). Where the voluminous stroma is enriched with myofibroblasts or smooth muscle cells, mucous neck cells (fundus) or pre-pyloric gland cell precursors (antrum) dominate the epithelium, whereas foveolar cells elsewhere. The polyp gland lacks chief and parietal cells and thus resembles the antral gland. Polyp glands, however, lack pepsinogen C expression, rendering them distinctively abnormal. Below, a schematic depiction of the gross anatomy of the mouse and the human stomach.

may note that parietal cell loss is well described to disrupt both gland cell and foveolar cell differentiation.⁴⁸ We consider this mechanism of failing epithelial differentiation to be unlikely in the *Lkb1*^{+/-} mice, for three reasons: i) the parietal cell defects in the fundus were limited to polyps, whereas peptic cell alterations were seen in other predisposed epithelium (Figure 2, Figure 3); ii) gland cell differentiation was disrupted also in the antral mucosa,

independently of fundic polyps; iii) no defects in foveolar cell differentiation were observed. Instead we propose a mechanism, through which global forward differentiation of the gland cell lineages ceases before the cells have specialized in their secretory functions.

With a sudden loss of parietal cells one would also have expected a process of antralization to drive dedifferentiation in the fundic mucosa.⁴² That is, fundic Peutz-Jeghers polyps would have contained antral metaplasia/spasmodic polypeptide-expressing metaplasia, characterized by larger than normal epithelial cells co-expressing TFF2 and IF in separate granules.⁴³ This we did not observe. The normal distribution of gastrin expression also argued against antralization, and the site-appropriate patterns of AB-PAS and Cdx2 staining gave no support for metaplastic differentiation in neither stomach nor intestinal polyps. By contrast these studies revealed a defect also in Paneth cell differentiation in intestinal polyps, revealing a more general disruption of epithelial differentiation in Peutz-Jeghers polyposis and suggesting similarities in the differentiation programs of the downward migrating exocrine cells of the stomach and small intestine. Curiously, Paneth cell differentiation is also disrupted on complete ablation of *Lkb1* in intestinal epithelium before a larger disruption of epithelial architecture and lethality.⁴⁹

Contrary to current understanding,^{6,34,50} this study demonstrated that Peutz-Jeghers polyps do not contain all epithelial cell types and lineages native to their site of occurrence. Some epithelial alterations were seen also in unaffected *Lkb1*^{+/-} mucosa, however, the Peutz-Jeghers and incipient polyp epithelium was much less differentiated and more proliferative. This is in agreement with a recent report of increased proliferation in intestinal Peutz-Jeghers patient polyps,⁵¹ which also described an expansion of the proliferative zone in unaffected small intestinal epithelium. We saw no such expansion in unaffected *Lkb1*^{+/-} stomach epithelium. However, we performed the analysis in a different setting, before the onset of macroscopic polyposis in young mice to identify alterations that would not be secondary to polyposis.

The discovery of defective peptic cell differentiation in Peutz-Jeghers polyps gives the opportunity to hypothesize that differentiation-promoting agents might slow polyp growth. Interestingly, glucocorticoids both induce peptic cell differentiation²⁶⁻²⁸ and reduce cyclooxygenase-2 expression,⁵² which previously has been noted to suppress PJS polyposis.³⁶

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