The absence of LPA receptor 2 reduces the tumorigenesis by Apc<sup>Min</sup> mutation in the intestine

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Lin S, Lee S, Shim H, Chun J, Yun CC. The absence of LPA receptor 2 reduces the tumorigenesis by Apc<sup>Min</sup> mutation in the intestine. Am J Physiol Gastrointest Liver Physiol 299: G1128–G1138, 2010. First published August 19, 2010; doi:10.1152/ajpgi.00321.2010.—Lysophosphatidic acid (LPA) is a lipid mediator that mediates several effects that promote cancer progression. The LPA receptor type 2 (LPA<sub>2</sub>) expression is often elevated in several types of cancers, including colorectal cancer (CRC). In this study, we investigated the role of LPA<sub>2</sub> in the development of intestinal adenomas by comparing Apc<sup>Mins</sup>/Lpar2<sup>−/−</sup> mice with Apc<sup>Mins</sup>/Lpar2<sup>+/−</sup> mice. There were 50% fewer intestinal adenomas in Apc<sup>Mins</sup>/Lpar2<sup>−/−</sup> mice than Apc<sup>Mins</sup>/Lpar2<sup>+/−</sup> mice. Smaller-size adenomas (<1 mm) were found at higher frequencies in Apc<sup>Mins</sup>/Lpar2<sup>−/−</sup> mice compared with Apc<sup>Mins</sup>/Lpar2<sup>+/−</sup> mice at the two age groups examined. The expression level of LPA<sub>2</sub> correlated with increased size of intestinal adenomas. Reduced tumor multiplicity and size in Apc<sup>Mins</sup>/Lpar2<sup>−/−</sup> mice correlated with decreased proliferation of intestinal epithelial cells. Apc<sup>Mins</sup>/Lpar2<sup>+/−</sup> mice showed increased levels of apoptosis, suggesting that LPA<sub>2</sub>-mediated signaling stimulates intestinal tumor development and progress by regulating both cell proliferation and survival. In addition, the expression levels of Krüppel-like factor 5 (KLF5), β-catenin, cyclin D1, c-Myc, and hypoxia-inducible factor-1α (HIF-1α) were significantly altered in Apc<sup>Mins</sup>/Lpar2<sup>−/−</sup> mice compared with Apc<sup>Mins</sup>/Lpar2<sup>+/−</sup> mice. In vitro studies using HCT116 cells showed that LPA induced cyclin D1, c-Myc, and HIF-1α expression, which was attenuated by knockdown of LPA2. In summary, intestinal tumor initiated by Apc mutations is altered by LPA2-mediated signaling, which regulates tumor growth and survival by altering multiple targets.

lyosphosphatidic acid; multiple intestinal neoplasia; familial adenomatous polyposis; adenomatous polyposis coli

COLORECTAL CANCER (CRC) is the third leading cause of cancer mortality in the United States. In >80% of sporadic and hereditary colon cancers, such as familial adenomatous polyposis (FAP), the tumor suppressor adenomatous polyposis coli (Apc) is mutated (19). Mutation in the Apc gene is an early event that stabilizes β-catenin in the cytoplasm and mobilizes β-catenin to the nucleus, where it forms β-catenin/T cell factor (TCF) complexes that activate oncogenic target genes such as c-Myc, c-Jun, and cyclin D1 (30, 34). FAP is modeled by the multiple intestinal neoplasia (Apc<sup>Mins</sup>/Lpar2<sup>+/−</sup>) mouse, which has one wild type and one truncation mutation at codon 850 of the Apc allele (32). However, unlike human FAP, the mouse model shows a much higher prevalence of adenomas in the small intestine (32).

Lysophosphatidic acid (LPA) is a pleiotropic lipid mediator that elicits its effect through a family of at least five G protein-coupled receptors, LPA<sub>1–4</sub> (2). LPA has been implicated in cancer because of its ability to stimulate cell proliferation, motility, survival, and invasion, including effects through β-catenin (28, 46). Subsequent reports that LPA is present at elevated levels in ascites of patients with ovarian cancer have provided a potential pathophysiologic local linkage between LPA and human cancer (27, 48). In addition, it has been shown that the LPA<sub>2</sub> receptor is overexpressed in ovarian, breast, and colon cancer (21, 41, 48, 51). Transgenic expression of LPA<sub>2</sub> in mouse ovaries resulted in increased expression of angiogenic factors (14). More recently, transgenic mice expressing each of LPA<sub>1</sub>, LPA<sub>2</sub>, and LPA<sub>3</sub> receptors or autotoxin, a key enzyme in LPA production from lysophosphatidylcholine, developed invasive and metastatic breast cancer (25). We previously showed that mice with targeted deletion of LPA<sub>2</sub> receptor expression, Lpar2<sup>−/−</sup>, are resistant to developing colitis-associated colon cancer induced with a series of azoxymethane (AOM) and dextran sodium sulfate (DSS) treatment (23). The reduced tumor burden in Lpar2<sup>−/−</sup> mice paralleled reduced inflammatory responses in the colon. Inflammation is considered a risk factor for many common malignancies, including cancers of the colon. Patients with inflammatory bowel disease (IBD) represent only a small fraction of CRC cases (1–2%), but the risk of CRC greatly increases with prolonged colitis, from ~1–2% at 10 years to 18% at 30 years of disease (6). However, the genetic basis for the increased risk of CRC in IBD patients and sporadic CRC differs. For example, mutations in the Apc/β-catenin pathway are infrequent and usually occur late in colitis-associated CRC. On the other hand, p53 mutations are much more frequent, with an early onset in colitis-associated CRC, whereas the occurrence of a p53 mutation is generally a late event in sporadic CRC (35). In this study, we assessed genetic interaction between Lpar2 and Apc<sup>Mins</sup>/Lpar2<sup>+/−</sup> in the promotion of intestinal tumorigenesis.

MATERIALS AND METHODS

Animals. Founder C57BL/6 mice heterozygous for the LPA<sub>2</sub> receptor allele (Lpar2<sup>+/−</sup>) were previously developed (23). Founder C57BL/6 male mice heterozygous for the Apc allele (Apc<sup>Mins</sup>/+) were purchased from the Jackson Laboratory. Apc<sup>Mins</sup>/+ mice were mated with Lpar2<sup>+/−</sup> females to obtain male mice heterozygous for both alleles (Apc<sup>Mins</sup>/Lpar2<sup>−/−</sup>). These mice were subsequently mated with Lpar2<sup>−/−</sup> females to generate wild-type (WT = Lpar2<sup>+/+</sup>), Lpar2<sup>−/−</sup>, Apc<sup>Mins</sup>/+, and Apc<sup>Mins</sup>/Lpar2<sup>−/−</sup> mice. Animals were maintained under the institutional guidelines of and the study was approved by the Emory University Animal Care and Use Committee.

Tumor assessment. At 15 or 21 wk of age, WT, Lpar2<sup>−/−</sup>, Apc<sup>Mins</sup>/+, and Apc<sup>Mins</sup>/Lpar2<sup>−/−</sup> mice were killed by CO2 asphyxiation. The entire small intestine and colon were dissected longitudinally and washed in PBS. Intestinal tissues were examined under a dissecting microscope in a blinded...
manner for the presence of adenomas. Adenomas were grouped by size: <1, 1–2, 2–3, and >3 mm.

Immunohistochemistry. Immunohistochemical staining of intestinal tissues was performed as described previously (23). Briefly, mouse intestinal tissues embedded in paraffin were cut into 5-μm sections. Sections were deparaffinized and rehydrated, and antigen unmasking was performed through microwave treatment in a citrate buffer. Vector Laboratories Avidin/Biotin Blocking kit was used in conjunction with a blocking buffer to reduce background and nonspecific secondary antibody binding. Sections were then stained for Ki67 (Leica), Krüpple-like factor 5 (KLF5; see Ref. 52), β-catenin (BD Biosciences), cyclin D1 (Biocare), c-Myc (Abcam), cleaved caspase-3 (Cell Signaling), and hypoxia-inducible factor-1α (HIF-1α; Novus Biological). Detection of primary antibodies and color development was done using the Dako (k6090) kit (Dako). Sections were then counterstained with hematoxylin, dehydrated, and covered with a cover slip. Images were acquired using an Axioskop 2 plus microscope equipped with an AxioCam MRc5 CCD camera (Zeiss).

Detection of hypoxia. Hypoxyprobe-1 (pimonidazole) kit (HPI) was used to detect intestinal hypoxia in vivo. Mice were given hypoxyprobe solution (60 mg/kg ip) or PBS as vehicle control as previously described (10). After the injection (3 h), animals were killed, and intestinal sections were embedded in paraffin. Paraffin-embedded sections were deparaffinized and prepared for immunohistochemical analysis as described earlier. Intestinal sections were incubated with Hypoxyprobe-1 PAb2627 rabbit antisera according to the manufacturer’s instruction.

Knockdown of LPA2 expression. HCT116 cells were transfected with pLKO.1-based RNA interference (RNAi) to express short-hairpin RNAs (shRNAs) targeting human Lpar2 gene (Sigma). As a control, the same plasmid containing a scrambled shRNA was used. After transfection (24 h), cells were serum deprived for 16–24 h and then treated with LPA or carrier. The efficacy of gene silencing of LPA2 was determined by reverse transcriptase-PCR using a primer set specific for LPAR2.

Quantitative RT-PCR. Mouse intestine was cut longitudinally and rinsed with cold PBS, and intestinal adenomas were dissected under a dissecting microscope and grouped according to the sizes. Isolated adenomas were snap-frozen in liquid nitrogen and stored at −80°C until needed. Total RNA was isolated from intestinal tissues using TRizol (Invitrogen), and cDNA was subsequently synthesized using the First Strand Synthesis kit (Invitrogen). Quantitative RT-PCR was performed as previously described (23) using SYGR Green Supermix (Bio-Rad) on the Eppendorf Mastercycler replex. The primers are as follows: LPA1, 5'-acaccagcctgacagcttct-3' and 5'-ctgtagaggggtgccatgtt-3'; LPA2, 5'-tcactggtcaatgcagtggt-3' and 5'-aagggtggagtccatcagtg-3'; LPA3, 5'-agggctcccatgaagctaat-3' and 5'-ttcat-

Fig. 1. Intestinal adenomas in ApcMin/mice in which familial adenomatous polyposis (FAP) is modeled by the multiple intestinal neoplasia/Lpar2−/− [founder C57BL/6 mice heterozygous for the lysophosphatidic acid receptor type 2 (LPA2) allele] mice. Mice were killed at the age of 15 or 21 wk, and the no. of adenomas of the two age groups was counted. A: comparison of the no. of intestinal adenomas/animal in two age groups of ApcMin/mice, *P < 0.01. B: comparison of the no. of colonic adenomas/mouse, *P < 0.01. C: graph showing the distribution of adenomas by adenoma size in ApcMin+ and ApcMin/Lpar2−/− mice at the age of 15 or 21 wk.
gaaggtagagcaag-3'; LPA4, 5'-tccatcagttgactgttt-3' and 5'-gaagcttcaagagcactgg-3'; LPA5, 5'-cactcagctctgacact-3' and 5'-ggagatgcagaggtaaga-3'; and 18S, 5'-gcaattacccgtgagc-3' and 5'-gcctcactaaccctac-3'.

Western immunoblot. Cell lysates were prepared in 1× Cell Lysis Buffer (Cell Signaling), and equal amounts of cell lysates were resolved by SDS-PAGE. Western immunoblot analysis was performed as previously described (51, 52) using an antibody against HIF-1α or glucose transporter 1 (GLUT1; Abcam). The expression levels of these proteins were quantified by normalizing the expression level of β-actin.

Statistical analysis. Statistical significance was assessed by one-way ANOVA or unpaired Student's t-tests using the SPSS 17 (SPSS). Data are expressed as means ± SE. The limit of statistical significance was set at P < .05.

RESULTS

The absence of LPA2 expression decreased tumor progression in ApcMin/+ mice. To investigate the role of LPA2 in FAP, we crossed Lpar2−/− mice with the ApcMin/+ mice, the well-established mouse model of FAP (32). We compared the number, size, and location of the adenomas that developed in ApcMin/+ and ApcMin+/−/Lpar2−/− mice at the age of 15 and 21 wk. ApcMin/+ mice developed 55.6 ± 9.6 intestinal adenomas/mouse (n = 7) at 15 wk. In comparison, the average number of adenomas in ApcMin+/−/Lpar2−/− mice was 28.1 ± 3.5 (n = 12; Fig. 1A), 51% less than ApcMin/+ mice (P < 0.01 by 2-tailed t-test). At 21 wk, an equivalent difference in average numbers of intestinal adenomas was found between ApcMin/+ mice (n = 10) and ApcMin+/−/Lpar2−/− mice (n = 16) (56.6 ± 3.7 vs. 24.6 ± 3.6, respectively). Neither WT nor Lpar2−/− mice developed adenomas in this period. ApcMin/+ mice develop fewer tumors in the colon (32); nonetheless, there was a statistical difference in the average number of colonic adenomas in the two genotypes (Fig. 1B): 2.4 ± 0.4 at 15 wk and 2.1 ± 0.4 at 21 wk for ApcMin/+ mice; 0.9 ± 0.4 at 15 wk and 0.9 ± 0.4 at 21 wk for ApcMin+/−/Lpar2−/− mice. We also examined whether there were any differences in the distribution of tumors of different sizes between ApcMin/+ and ApcMin+/−/Lpar2−/− mice. Figure 1C shows the shift in the tumor size distribution from age 15 to 21 wk, with the mode for both genotypes increasing from <1 to 1–2 mm. However, there were more tumors of larger sizes (>2 mm) in ApcMin+/−/Lpar2−/− compared with ApcMin+/−/Lpar2−/− mice. These results suggest that the absence of the Lpar2 allele affects tumor growth as well as tumor incidence.

The expression level of LPA2 receptor is associated with increased adenomas. It has been shown previously that LPA2 expression is elevated in human CRC patients and colon cancer cell lines (41, 51). In our previous study, we found an elevated LPA2 mRNA level in intestinal adenomas of ApcMin/+ mice compared with normal intestinal tissue (23). However, we revisited this issue to determine whether the change in LPA2 mRNA expression is associated with the increase in adenoma size. As shown previously (24), the relative expression level of LPA2 mRNA is low compared with that of LPA1 or LPA3 (Fig. 2A). Importantly, a gradual increase in LPA2 mRNA expression with increased tumor size was observed with
adenomas >3 mm in size displaying the highest level of LPA2 mRNA expression (Fig. 2B). Unlike LPA2 mRNA, the mRNA expression of LPA1, LPA4, and LPA5 was not changed, whereas LPA3 mRNA expression was lower in intestinal adenomas compared with the control (Fig. 2A).

Cell proliferation is decreased in ApcMin-/Lpar2-/- mice. To further understand the cellular basis for the differences in tumor progression in ApcMin-/Lpar2-/- mice, we examined epithelial cell proliferation by immunohistological staining of Ki67. In WT and Lpar2-/- mice (Fig. 3A), Ki67 labeling was confined to the nuclei of epithelial cells in the proliferative compartment at the bottom of intestinal crypts, and no difference was observed between WT and Lpar2-/- mice, as previously reported (23). Similar patterns were observed in healthy intestinal tissues from ApcMin-/+ and ApcMin-/+ /Lpar2-/- mice (Fig. 3B, left). However, a significant difference was observed when tumors from ApcMin-/+ or ApcMin-/+ /Lpar2-/- mice were compared (Fig. 3B, right). In ApcMin-/+ mice, Ki67 labeling extended from the base of the tumor to the surface of the lumen. On the contrary, Ki67-positive epithelial cells were largely limited to the base region of the tumor in ApcMin-/+ /Lpar2-/- mice, further substantiating that LPA2-mediated signaling potentiates proliferation of epithelial cells in the intestinal tract.

The level of apoptosis is increased in ApcMin-/+ /Lpar2-/- mice. We have shown previously that LPA2-mediated signaling protects human colon cancer cells from chemically induced apoptosis (37). An immunohistological staining for cleaved caspase-3 in the small intestinal tissues from WT, Lpar2-/-, ApcMin-/-, and ApcMin-/+ /Lpar2-/- mice was performed to evaluate the level of epithelial cell apoptosis. The absence of LPA2 does not alter the level of apoptosis in the mouse intestine (data not shown). Similarly, no discernable difference was observed between the normal-looking intestinal tissues of ApcMin-/+ and ApcMin-/+ /Lpar2-/- mice (Fig. 3C). On the other hand, fewer apoptotic cells were found in tumors of ApcMin-/+ mice, consistent with the previous report that Apc regulates apoptosis (31). Compared with ApcMin-/+ mice, increased staining for cleaved caspase-3 was observed in the tumors of ApcMin-/+ /Lpar2-/- mice (Fig. 3, C and D).

KLF5 and β-catenin expression is altered in ApcMin-/+ /Lpar2-/- mice. Previous studies have identified KLF5 as a mediator of LPA-induced proliferation of colon cancer cells (23, 52); hence, we performed immunohistochemical staining

Fig. 3. Cell proliferation and apoptosis in ApcMin-/+ and ApcMin-/+ /Lpar2-/- mice. A: intestinal sections from WT and Lpar2-/- mice were immunolabeled using the antibodies against Ki67. Scale bar: 200 μm. B: intestinal sections from ApcMin-/+ and ApcMin-/+ /Lpar2-/- mice were immunolabeled using an antibody against cleaved caspase-3. Scale bar (in blue): 30 μm. C: intestinal sections from ApcMin-/+ and ApcMin-/+ /Lpar2-/- mice were immunolabeled using an anti-Ki67 antibody. Left, intestinal sections of normal-appearing ileum of 21-wk-old mice. Right, adenomatous lesions. Red arrows indicate cleaved caspase-3-positive cells. Scale bar (in red): 200 μm. Representative images are shown. D: no. of cells positive for cleaved caspase-3 in adenomas is shown; 5–6 microscopic views/section and 10 sections from 3 animals of each genotype were examined. *P < 0.01 by paired 2-tailed t-test.
Fig. 4. Krüppel-like factor 5 (KLF5) and β-catenin expression in Apc<sup>Min/+</sup>/Lpar2<sup>−/−</sup> mice compared with Apc<sup>Min/+</sup> mice. Intestinal sections of 21-wk-old Apc<sup>Min/+</sup> and Apc<sup>Min/+</sup>/Lpar2<sup>−/−</sup> mice were stained for KLF5 (A) and β-catenin (B). Left, intestinal sections of normal-appearing ileum of 21-wk-old mice. Right, adenomatous lesions. Scale bars: 200 μm (in red) for KLF5 and 50 μm (in green) for β-catenin. C: equal amounts of mouse intestinal lysates were analyzed by Western blotting for the expression levels of β-catenin. For Apc<sup>Min/+</sup> and Apc<sup>Min/+</sup>/Lpar2<sup>−/−</sup> mice, lysates were prepared from isolated adenomas. β-Actin expression was used as a loading control. Relative changes in β-catenin expression are indicated below the immunoblot. Representative images from 4 separate experiments are shown.
for KLF5. As shown previously (23, 42), KLF5 expression in the mouse intestine was not altered by the absence of LPA2 expression (data not shown), and, similarly, no significant difference was observed between the healthy intestinal tissues of ApcMin/+ and ApcMin+/Lpar2−/− mice (Fig. 4A). Whereas the tumors of ApcMin/+ mice showed heightened staining for KLF5 with the staining extending to the surface of tumor, KLF5 staining in the tumors of ApcMin+/Lpar2−/− mice was significantly weaker and largely limited to the cryptal regions.

It has been shown that LPA activates the β-catenin pathway via phosphorylation of glycogen synthase kinase 3β and nuclear translocation of β-catenin (8, 49). Thus we examined whether nuclear translocation of β-catenin is altered in the absence of LPA2 expression. Immunohistochemical staining of intestinal sections of ApcMin/+ mice showed prominent nuclear staining of β-catenin in epithelial cells within adenomatous tumors (Fig. 4B). Surprisingly, β-catenin staining in the tumors of ApcMin+/Lpar2−/− mice was primarily in the cytoplasm, and fewer cells had nuclear β-catenin staining compared with ApcMin/+ mice. On the contrary, the total expression level of β-catenin was not significantly altered based on Western blot (Fig. 4C), suggesting that the loss of LPA2 only modulates β-catenin translocation and not its expression.

To further investigate the mechanism of altered cell proliferation, we examined the expression of cyclin D1 and c-Myc. Again, the basal expression of cyclin D1 and c-Myc was not affected by the absence of LPA2 (data not shown) or before the onset of adenoma in ApcMin/+ mice (Fig. 5, A and B, left). On the contrary, the tumors of ApcMin+/Lpar2−/− mice exhibited reduced levels of cyclin D1 and c-Myc compared with ApcMin/+ mice. The differences in cyclin D1 and c-Myc staining for these genotypes were confirmed by Western immunoblotting where the expression levels of both cyclin D1 and c-Myc were markedly elevated in ApcMin/+ mice compared with ApcMin+/Lpar2−/− mice (Fig. 5C).

To ensure that cyclin D1 and c-Myc are downstream targets of LPA2-mediated signaling, LPA2-RNAi or control-RNAi transfected HCT116 cells were treated with 1 μM LPA. LPA induced expression of both cyclin D1 and c-Myc, whereas knockdown of LPA2 expression significantly attenuated the induction of cyclin D1 and c-Myc expression by LPA (Fig. 5D). These results indicate that the altered expression of KLF5, β-catenin, cyclin D1, and c-Myc provides the molecular basis for their role in tumor development.
for the reduced tumorigenesis in the intestine of Apc\textsuperscript{Min+/+} / Lpar2\textsuperscript{−/−} mice. 

Hypoxia is a hallmark of cancer, and HIF plays an essential role in cellular and systemic responses to hypoxia (38). It has been shown that LPA induces HIF-1α expression in ovarian cancer cells (17, 22), but a similar effect in colon cancer has not been investigated. As shown previously (44), HIF-1α is expressed at a relatively high level in the intestinal epithelial cells of WT and Lpar2\textsuperscript{−/−} mice without a notable difference between the two genotypes (data not shown). Likewise, HIF-1α expression of Apc\textsuperscript{Min+/+} and Apc\textsuperscript{Min+/+} / Lpar2\textsuperscript{−/−} mice looked alike. However, in the intestinal adenomas of Apc\textsuperscript{Min+/+} mice, HIF-1α staining was elevated compared with the normal epithelial cells of the same mice (Fig. 6, A and B). Interestingly, HIF-1α was localized more frequently in the nuclei of adenomatous cells of Apc\textsuperscript{Min+/+} mice, which is in contrast to WT and Lpar2\textsuperscript{−/−} mice where HIF-1α staining was largely in the cytoplasm with occasional staining in the nuclei (Fig. 6A).

In Apc\textsuperscript{Min+/+} / Lpar2\textsuperscript{−/−} mice, HIF-1α staining was relatively lower than in Apc\textsuperscript{Min+/+} mice, and less frequent nuclear HIF-1α staining was observed.

The difference in HIF-1α expression in these mice was corroborated by determining intestinal tissue oxygen gradient in vivo by using the bioreductive drug hypoxia marker pimonidazole. Intestinal epithelial cells were under varying degrees of hypoxia (Fig. 7), consistent with the expression pattern of HIF-1α. Importantly, hypoxia within the tumors of Apc\textsuperscript{Min+/+} / Lpar2\textsuperscript{−/−} mice was significantly attenuated compared with the intestinal adenomas of Apc\textsuperscript{Min+/+} mice. The differential levels of HIF-1α expression in the two genotypes were confirmed by Western immunoblotting of lysates from intestinal tumors. Figure 6, B and C shows that the expression level of HIF-1α is significantly elevated in Apc\textsuperscript{Min+/+} mice compared with Apc\textsuperscript{Min+/+} / Lpar2\textsuperscript{−/−} mice when either total intestinal mucosa lysates or individual tumors were compared.

To determine whether HIF-1α activity is altered in Apc\textsuperscript{Min+/+} / Lpar2\textsuperscript{−/−} mice relative to Apc\textsuperscript{Min+/+} mice, we examined the expression of a known HIF-1α downstream target, GLUT1, in the tumors of these mice. Consistent with the decreased HIF-1α expression, the expression level of GLUT1 was significantly lower in Apc\textsuperscript{Min+/+} / Lpar2\textsuperscript{−/−} mice compared with Apc\textsuperscript{Min+/+} mice (Fig. 6C, top and middle), indicating that HIF-1α activity in tumors is attenuated by the loss of LPA2 function.

**Fig. 6. Reduced hypoxia in Apc\textsuperscript{Min+/+} / Lpar2\textsuperscript{−/−} mice.** A: immunohistochemical labeling of hypoxia-inducible factor-1α (HIF-1α) was performed on paraffin-embedded intestinal sections of Apc\textsuperscript{Min+/+} and Apc\textsuperscript{Min+/+} / Lpar2\textsuperscript{−/−} mice. **Left** and **right**, representative images of normal-appearing ileum and adenomatous lesions from 21-wk-old mice, respectively. Scale bar: 50 μm. B: HIF-1α expression in total intestinal mucosal lysates was determined by Western blotting. β-Actin expression was used as a loading control. Relative changes in HIF-1α expression are indicated. C: 3 individual tumors (T1, T2, and T3) from Apc\textsuperscript{Min+/+} or Apc\textsuperscript{Min+/+} / Lpar2\textsuperscript{−/−} mice were isolated, and HIF-1α and glucose transporter 1 (GLUT1) expression in each tumor was determined. Relative changes in protein expression are indicated. D: the effect of LPA on HIF-1α expression in HCT116 cells was determined. Cells were treated with 1 μM LPA or carrier under normoxic or hypoxic conditions for 16 h. E: HCT116 cells transfected with control-RNAi or LPA2-RNAi were incubated with 1 or 10 μM LPA for 16 h. HIF-1α and β-actin protein expression levels were determined. A representative Western blot from 3 independent experiments is shown.
To attain direct evidence that LPA modulates HIF-1α expression in colon cancer cells, HCT116 cells were exposed to LPA. As shown in Fig. 6C, LPA induced HIF-1α expression in HCT116 cells. Induction of HIF-1α was seen as early as 3 h and reached the maximum at 16 h (data not shown). In addition, HIF-1α expression induced by hypoxia was further stimulated when LPA was supplemented in the media. Silencing of LPA2 expression markedly decreased the activation of HIF-1α expression (Fig. 6D), demonstrating that LPA2 is accountable for the induction of HIF-1α by LPA.

DISCUSSION

Apc plays a central role in the etiology of sporadic and hereditary CRC (1, 20). Loss of Apc function is an early event in the development of FAP as well as sporadic CRC. The ApcMin/+ mouse provides a model of colon tumorigenesis where Apc germ-line mutation results in the formation of adenomatous polyps (32). In this study, we demonstrated that the absence of LPA2 attenuates the progression of colon cancer in ApcMin/+ mice. The absence of LPA2 did not prevent development of adenomas, but a significant decrease in tumor multiplicity in ApcMin/+Lpar2−/− mice compared with ApcMin/+ mice was found. Therefore, the current finding, together with the observation that LPA2 expression is upregulated in CRC, provides compelling evidence that the LPA-LPA2 signaling axis is a significant tumor-promoting pathway in the intestinal tract.

Mice lacking LPA2 expression do not develop any apparent gross defect (4). However, studies in other cell types have demonstrated altered downstream signaling pathways as well as anti-apoptotic effects in the promotion of proliferating stem cell survival (4, 18). Furthermore, a challenge by a nonlethal dose of radiation or inflammation-inducing agents has revealed additional pathophysiological roles involving LPA2 (7, 23). In the current study, loss of LPA2 expression in the setting of Apc mutation reduced tumor incidence and size in mouse intestine. The differences in tumor progression between ApcMin/+ and ApcMin/+Lpar2−/− mice were attributed to two cellular effects: reduced proliferation and increased apoptosis. Cell proliferation and apoptosis are among multiple effects mediated by LPA in a variety of cells (2, 3, 9, 15). The pro-proliferative effect of LPA on colon cancer cells, such as DLD1, HCT116, and SW480 cells, has been demonstrated (40, 49, 52). The pro-proliferative effects of LPA on DLD1 cells is mediated by both LPA1 and LPA2, whereas LPA3, but not LPA1, promote proliferation of HCT116 and LS174T cells via nuclear
translocation of β-catenin (49). In addition, a body of evidence supports the role of LPA as a survival factor that renders cancer cells resistant to apoptosis-inducing treatments (28, 29). LPA rescued Caco-2 cells from apoptosis elicited by a chemotherapeutic drug, and Lpar2−/− mice showed a significantly increased rate of radiation-induced apoptosis and less crypt survival (7, 37).

Aberrant expression of LPA2 mRNA in human adenocarcinomas and colon cancer cell lines has been demonstrated (41, 51). Nonetheless, it is interesting that the expression level of LPA2 mRNA correlated with increasing sizes of adenomas, raising the possibility that the increased LPA2 level helps to potentiate tumorigenic transformation in the intestine.

LPA stimulates proliferation of colon cancer cells in part through cross talk with the Apc/β-catenin pathway (49). However, the prevalence of a mutation in the Apc gene in FAP and sporadic CRC calls for an alternative pathway for proliferation of colon cancer cells potentially independent of Apc/β-catenin. We showed consequently that LPA induces KLF5 expression in both normal intestinal and colon cancer cells (52). The induction of KLF5 was observed in colon tumors induced by AOM and DSS, which was attenuated in Lpar2−/− mice (23). Consistently, decreased epithelial proliferation in ApcMin+/−/Lpar2−/− mice correlated with reduced expression of KLF5. We initially proposed that LPA independently activates β-catenin and KLF5 (52). However, it was shown recently that KLF5 physically interacts with β-catenin to enhance the nuclear localization and transcriptional activity of β-catenin, suggesting that the induction of KLF5 by LPA2 might further foster LPA-induced gene expression and proliferation by enhancing β-catenin nuclear translocation (26).

Accumulation of β-catenin in the nuclei through the formation of β-catenin/TCF complexes activates downstream targets, such as c-Myc and cyclin D1 (13, 45). Previous studies showed that LPA acting on LPA2 induced nuclear translocation of β-catenin in HCT116 or LS174T cells with WT Apc and β-catenin but not in SW480 cells with mutated Apc and β-catenin (49, 52). Moreover, we reported recently that, in mouse inflammation-associated colon cancer induced by AOM and DSS, β-catenin expression was decreased in tumors of Lpar2−/− mice colon relative to WT colon, but β-catenin nuclear localization was not significantly perturbed by the absence of LPA2 (23). In light of these reports, it was unexpected to find that the nuclear translocation of β-catenin was impeded in ApcMin+/−/Lpar2−/− mice. The conventional model of Apc function predicts the nuclear accumulation of β-catenin upon Apc mutation, but recent evidence suggests that loss of Apc function might be insufficient for β-catenin nuclear localization and requires additional activation of K-Ras or Rac1 (33, 47). Interestingly, epidermal growth factor (EGF) signaling could support the nuclear accumulation of β-catenin in the absence of K-Ras mutation (33). LPA is a potent trans-activator of EGF receptor (EGFR) (5), and it is conceivable that transactivation of EGFR by LPA2-mediated signaling facilitates nuclear translocation of β-catenin. As such, the absence of LPA2 attenuates signaling by EGFR and hence hampers β-catenin activation in ApcMin+/−/Lpar2−/− mice. Then, how do we explain that the loss of LPA2 function did not alter β-catenin nuclear translocation in AOM/DSS-induced colon cancer (23)? AOM-induced lesions are frequently associated with K-Ras mutations, and evidence supports the presence of β-catenin mutations in rats and mice exposed to AOM (36, 43). Hence, putative mutations in K-Ras and β-catenin in Lpar2−/− mice exposed to AOM and DSS might have been sufficient for nuclear targeting of β-catenin independent of LPA2 expression.

Hypoxia occurs during acute and chronic diseases, including cancer, and it is associated with tumor progression, angiogenesis, and resistance to radiation therapy and chemotherapy (12). Among several hypoxic genes, Hif-1α and Hif-2α are considered the primary targets. It was shown that LPA-induced secretion of vascular endothelial growth factor and invasion of ovarian cancer cells are enhanced by induction of HIF-1α by LPA (17, 22). We demonstrated that LPA is a potent inducer of HIF-1α in colon cancer cells under both normoxic and hypoxic conditions. Moreover, the absence of LPA2 expression in ApcMin+/− mice significantly decreased the levels of hypoxia in intestinal adenomas as determined by HIF-1α expression and with the chemical marker of hypoxia primonidazole. The roles of HIF in intestinal mucosa are complex. Studies have shown that HIF-1α and HIF-2α have seemingly different roles in the setting of colitis (16, 39). The presence of HIF-1α in normal mucosa and adenocarcinomas of the human colon has been shown (11, 50). Moreover, both HIF-1α and HIF-2α have a significant impact on survival of CRC patients, although HIF-2α expression showed a better correlation with tumor angiogenesis in the colon (11, 50). Although our analysis in the current study was on HIF-1α expression, LPA also induced the expression of HIF-2α (an unpublished observation).

In summary, the absence of LPA2 significantly attenuated the initiation and progression of tumor generation via multiple pathways in a mouse model of FAP. Together with the role of LPA2 in colitis-associated colon cancer, our results herein highlight the importance of LPA2 in intestinal tumorigenesis and support for development of LPA2-specific agents as part of therapeutic implementation to prevent and treat CRC.

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DISCLOSURES

The authors have no conflicts of interest.

REFERENCES

LPA stimulates multiple signaling pathways by activating G protein-coupled lysophosphatidic acid receptors. The receptors are divided into two main types: LPA1 and LPA2. LPA1, expressed predominantly in the colon, mediates proliferative responses, while LPA2 interacts with specific G protein-coupled receptors and mediates tumor angiogenesis. LPA2 modulation is critical in colorectal cancer development and progression. The receptor's role in cancer is highlighted by the fact that its expression is upregulated in colorectal tumors. Additionally, LPA2 inhibitors can suppress tumor formation in experimental models. The importance of LPA2 in cancer envisages its potential as a diagnostic and therapeutic target.

