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The absence of LPA receptor 2 reduces the tumorigenesis by Apc^{Min} mutation in the intestine

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[1]*Division of Digestive Diseases, Departments of Medicine and* [2]*Radiology,* [3]*Winship Cancer Institute and* [4]*Department of Physiology, Emory University School of Medicine, Atlanta, Georgia; and* [5]*Department of Molecular Biology, Scripps Research Institute, La Jolla, California*

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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^{Min} 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 progress. The LPA receptor type 2 (LPA_2) expression is often elevated in several types of cancers, including colorectal cancer (CRC). In this study, we investigated the role of LPA_2 in the development of intestinal adenomas by comparing $Apc^{Min/+}$ mice with $Apc^{Min/+}/Lpar2^{-/-}$ mice. There were 50% fewer intestinal adenomas in $Apc^{Min/+}/Lpar2^{-/-}$ mice than $Apc^{Min/+}$ mice. Smaller-size adenomas (<1 mm) were found at higher frequencies in $Apc^{Min/+}/Lpar2^{-/-}$ mice compared with $Apc^{Min/+}$ mice at the two age groups examined. The expression level of LPA_2 correlated with increased size of intestinal adenomas. Reduced tumor multiplicity and size in $Apc^{Min/+}/Lpar2^{-/-}$ mice correlated with decreased proliferation of intestinal epithelial cells. $Apc^{Min/+}/Lpar2^{-/-}$ mice showed an increased level of apoptosis, suggesting that LPA_2-mediated signaling stimulates intestinal tumor development and progress by regulating both cell proliferation and survival. In addition, the expression levels of Krüpple-like factor 5 (KLF5), β-catenin, cyclin D1, c-Myc, and hypoxia-inducible factor-1α (HIF-1α) were significantly altered in $Apc^{Min/+}/Lpar2^{-/-}$ mice compared with $Apc^{Min/+}$ 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 LPA_2. In summary, intestinal tumor initiated by Apc mutations is altered by LPA_2-mediated signaling, which regulates tumor growth and survival by altering multiple targets.

lysophosphatidic 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 ($Apc^{Min/+}$) 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_1–LPA_5 (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 pathophysiolocal linkage between LPA and human cancer (27, 48). In addition, it has been shown that the LPA_2 receptor is overexpressed in ovarian, breast, and colon cancer (21, 41, 48, 51). Transgenic expression of LPA_2 in mouse ovaries resulted in increased expression of angiogenic factors (14). More recently, transgenic mice expressing each of LPA_1, LPA_2, and LPA_3 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_2 receptor expression, $Lpar2^{-/-}$, are resistant to developing colitis-associated colon cancer induced with a series of azoxymethasone (AOM) and dextran sodium sulfate (DSS) treatment (23). The reduced tumor burden in $Lpar2^{-/-}$ 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 $Apc^{Min/+}$ in the promotion of intestinal tumorigenesis.

MATERIALS AND METHODS

Animals. Founder C57BL/6 mice heterozygous for the LPA_2 receptor allele ($Lpar2^{+/-}$) were previously developed (23). Founder C57BL/6 male mice heterozygous for the *Apc* allele ($Apc^{Min/+}$) were purchased from the Jackson Laboratory. $Apc^{Min/+}$ males were mated with $Lpar2^{+/-}$ females to obtain male mice heterozygous for both alleles ($Apc^{Min/+}/Lpar2^{+/-}$). These mice were subsequently mated with $Lpar2^{+/-}$ females to generate wild-type (WT = $Lpar2^{+/+}$), $Lpar2^{-/-}$, $Apc^{Min/+}$, and $Apc^{Min/+}/Lpar2^{-/-}$ 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, $Lpar2^{-/-}$, $Apc^{Min/+}$, and $Apc^{Min/+}/Lpar2^{-/-}$ mice were killed by CO_2 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

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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 (Zeiss) 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 LPA$_2$ 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 LPA$_2$ 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 iQ SYGR Green Supermix (Bio-Rad) on the Eppendorf Mastercycler realplex. The primers are as follows: LPA$_1$:,5′-acaccagcctgacagcttct-3′ and 5′-ctgtagagggggtgccatgtt-3′; LPA$_2$, 5′-tcactggtcaatgcagtggt-3′ and 5′-aagggtggagtccatcagtg-3′; LPA$_3$, 5′-agggctcccatgaagctaat-3′ and 5′-ttcat-

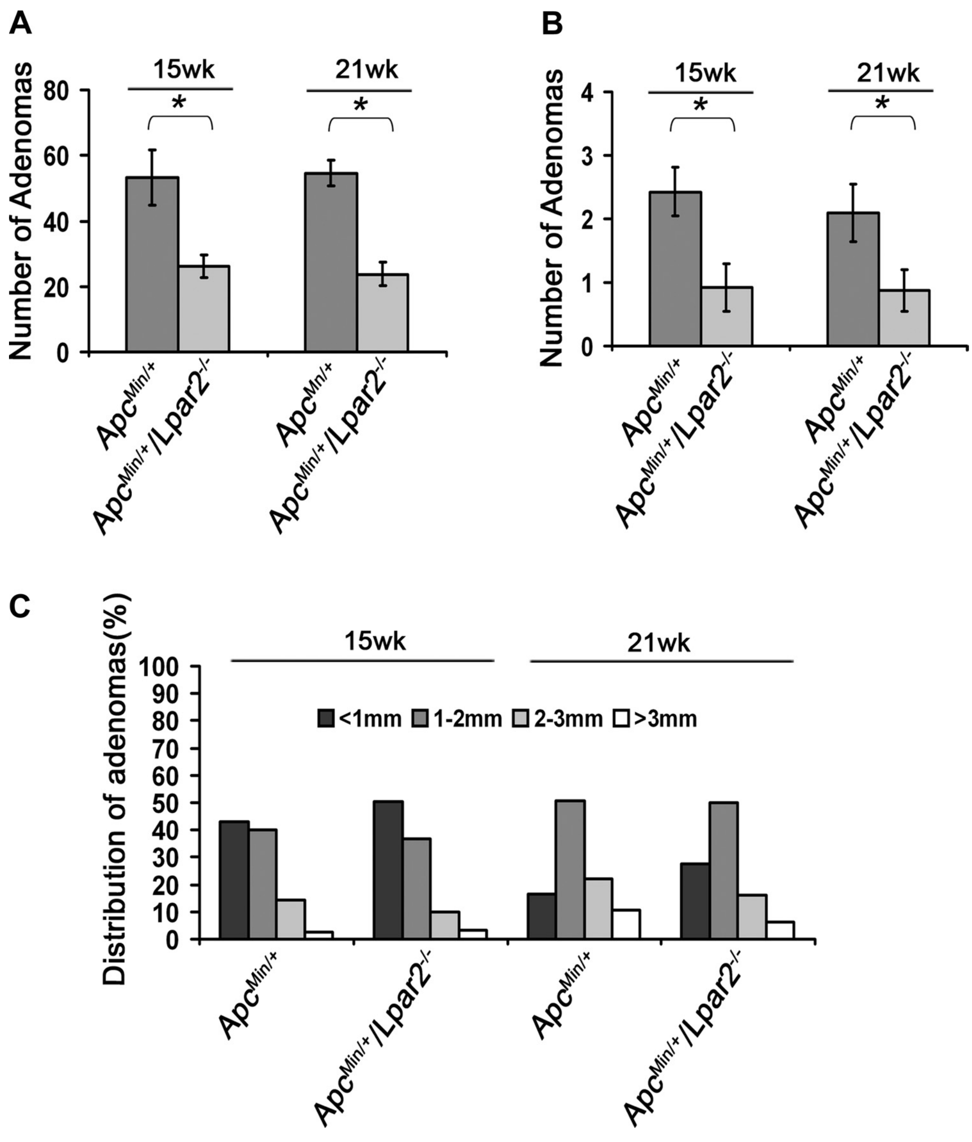

Fig. 1. Intestinal adenomas in *Apc*$^{Min/+}$ [mice in which familial adenomatous polyposis (FAP) is modeled by the multiple intestinal neoplasia]/*Lpar2*$^{-/-}$ [founder C57BL/6 mice heterozygous for the lysophosphatidic acid receptor type 2 (LPA$_2$) allele] mice. Mice were killed at the age of 15 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 *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ 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 *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice at the age of 15 or 21 wk.

gacggagttgagcag-3′; LPA$_4$, 5′-tgcatcagtgtggatcgttt-3′ and 5′-gaagccttcaaagcaagtgg-3′; LPA$_5$, 5′-gctccagtgccctgactatc-3′ and 5′-gggaagtgacagggtgaaga-3′; and 18S, 5′-gcaattattccccatgaacg-3′ and 5′-ggcctcactaaaccatccaa-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 cyclin D1, c-Myc, 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 LPA$_2$ expression decreased tumor progression in Apc$^{Min/+}$ mice. To investigate the role of LPA$_2$ in FAP, we crossed *Lpar2*$^{-/-}$ mice with the *Apc*$^{Min/+}$ mice, the well-established mouse model of FAP (32). We compared the number, size, and location of the adenomas that developed in *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice at the age of 15 and 21 wk. *Apc*$^{Min/+}$ mice developed 55.6 ± 9.6 intestinal adenomas/mouse ($n = 7$) at 15 wk. In comparison, the average number of adenomas in *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice was 28.1 ± 3.5 ($n = 12$; Fig. 1*A*), 51% less than *Apc*$^{Min/+}$ mice (P <0.01 by 2-tailed *t*-test). At 21 wk, an equivalent difference in average numbers of intestinal adenomas was found between *Apc*$^{Min/+}$ mice ($n = 10$) and *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice ($n = 16$) (56.6 ± 3.7 vs. 24.6 ± 3.6, respectively). Neither WT nor *Lpar2*$^{-/-}$ mice developed adenomas in this period. *Apc*$^{Min/+}$ 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. 1*B*): 2.4 ± 0.4 at 15 wk and 2.1 ± 0.4 at 21 wk for *Apc*$^{Min/+}$ mice; 0.9 ± 0.4 at 15 wk and 0.9 ± 0.4 at 21 wk for *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice. We also examined whether there were any differences in the distribution of tumors of different sizes between *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice. Figure 1*C* 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 *Apc*$^{Min/+}$ compared with *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice. These results suggest that the absence of the *Lpar2* allele affects tumor growth as well as tumor incidence.

The expression level of LPA$_2$ receptor is associated with increased adenomas. It has been shown previously that LPA$_2$ expression is elevated in human CRC patients and colon cancer cell lines (41, 51). In our previous study, we found an elevated LPA$_2$ mRNA level in intestinal adenomas of *Apc*$^{Min/+}$ mice compared with normal intestinal tissue (23). However, we revisited this issue to determine whether the change in LPA$_2$ mRNA expression is associated with the increase in adenoma size. As shown previously (24), the relative expression level of LPA$_2$ mRNA is low compared with that of LPA$_1$ or LPA$_5$ (Fig. 2*A*). Importantly, a gradual increase in LPA$_2$ mRNA expression with increased tumor size was observed with

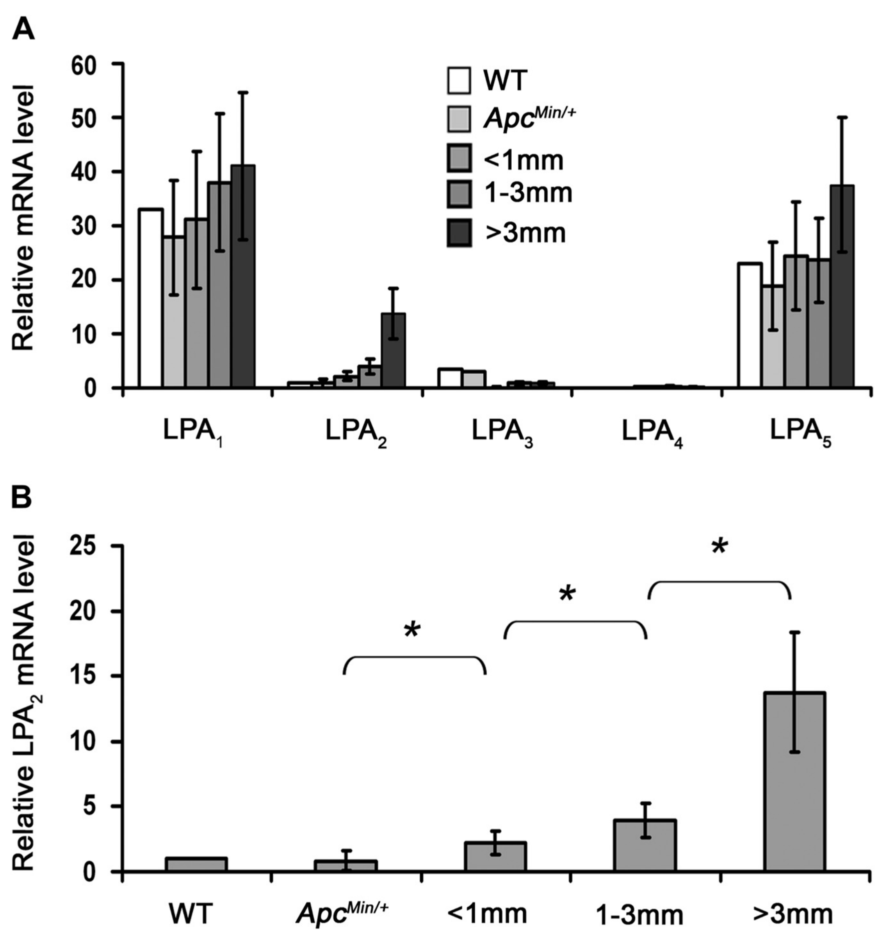

Fig. 2. The expression of lysophosphatidic acid (LPA) receptors in intestinal adenomas of *Apc*$^{Min/+}$ mice. Adenomas were isolated under a dissecting microscope and grouped according to their sizes. As controls, the healthy ileal epithelial cells from wild-type (WT) and *Apc*$^{Min/+}$ mice were isolated and processed. Total RNA was prepared for quantitative RT-PCR to determine the mRNA levels of LPA receptors. *A*: expression levels of LPA receptor mRNAs are shown. The mRNA level of each LPA receptor was normalized to the 18S mRNA level of the same sample. The relative expression levels are expressed as fold changes relative to the LPA$_2$ mRNA level in the intestinal epithelia of WT (=*Lpar2*$^{+/+}$) mice; $n = 6$. *B*: the expression levels of LPA$_2$ mRNA in WT and adenomas of *Apc*$^{Min/+}$ mice are shown. LPA$_2$ mRNA expression level was normalized to 18S mRNA expression; $n = 6$. *$P < 0.01$ between two groups of adenomas.

adenomas >3 mm in size displaying the highest level of LPA$_2$ mRNA expression (Fig. 2*B*). Unlike LPA$_2$ mRNA, the mRNA expression of LPA$_1$, LPA$_4$, and LPA$_5$ was not changed, whereas LPA$_3$ mRNA expression was lower in intestinal adenomas compared with the control (Fig. 2*A*).

Cell proliferation is decreased in Apc$^{Min/+}$/Lpar2$^{-/-}$ mice. To further understand the cellular basis for the differences in tumor progression in *Apc$^{Min/+}$/Lpar2$^{-/-}$* mice, we examined epithelial cell proliferation by immunohistological staining of Ki67. In WT and *Lpar2$^{-/-}$* mice (Fig. 3*A*), 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 *Apc$^{Min/+}$ and Apc$^{Min/+}$/Lpar2$^{-/-}$* mice (Fig. 3*B*, *left*). However, a significant difference was observed when tumors from *Apc$^{Min/+}$* or *Apc$^{Min/+}$/Lpar2$^{-/-}$* mice were compared (Fig. 3*B*, *right*). In *Apc$^{Min/+}$* 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 *Apc$^{Min/+}$/Lpar2$^{-/-}$* mice, further substantiating that LPA$_2$-mediated signaling potentiates proliferation of epithelial cells in the intestinal tract.

The level of apoptosis is increased in Apc$^{Min/+}$/Lpar2$^{-/-}$ mice. We have shown previously that LPA$_2$-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$^{-/-}$*, *Apc$^{Min/+}$*, and *Apc$^{Min/+}$/Lpar2$^{-/-}$* mice was performed to evaluate the level of epithelial cell apoptosis. The absence of LPA$_2$ 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 *Apc$^{Min/+}$* and *Apc$^{Min+/+}$/Lpar2$^{-/-}$* mice (Fig. 3*C*). On the other hand, fewer apoptotic cells were found in tumors of *Apc$^{Min/+}$* mice, consistent with the previous report that Apc regulates apoptosis (31). Compared with *Apc$^{Min/+}$* mice, increased staining for cleaved caspase-3 was observed in the tumors of *Apc$^{Min/+}$/Lpar2$^{-/-}$* mice (Fig. 3, *C* and *D*).

KLF5 and β-catenin expression is altered in Apc$^{Min/+}$/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

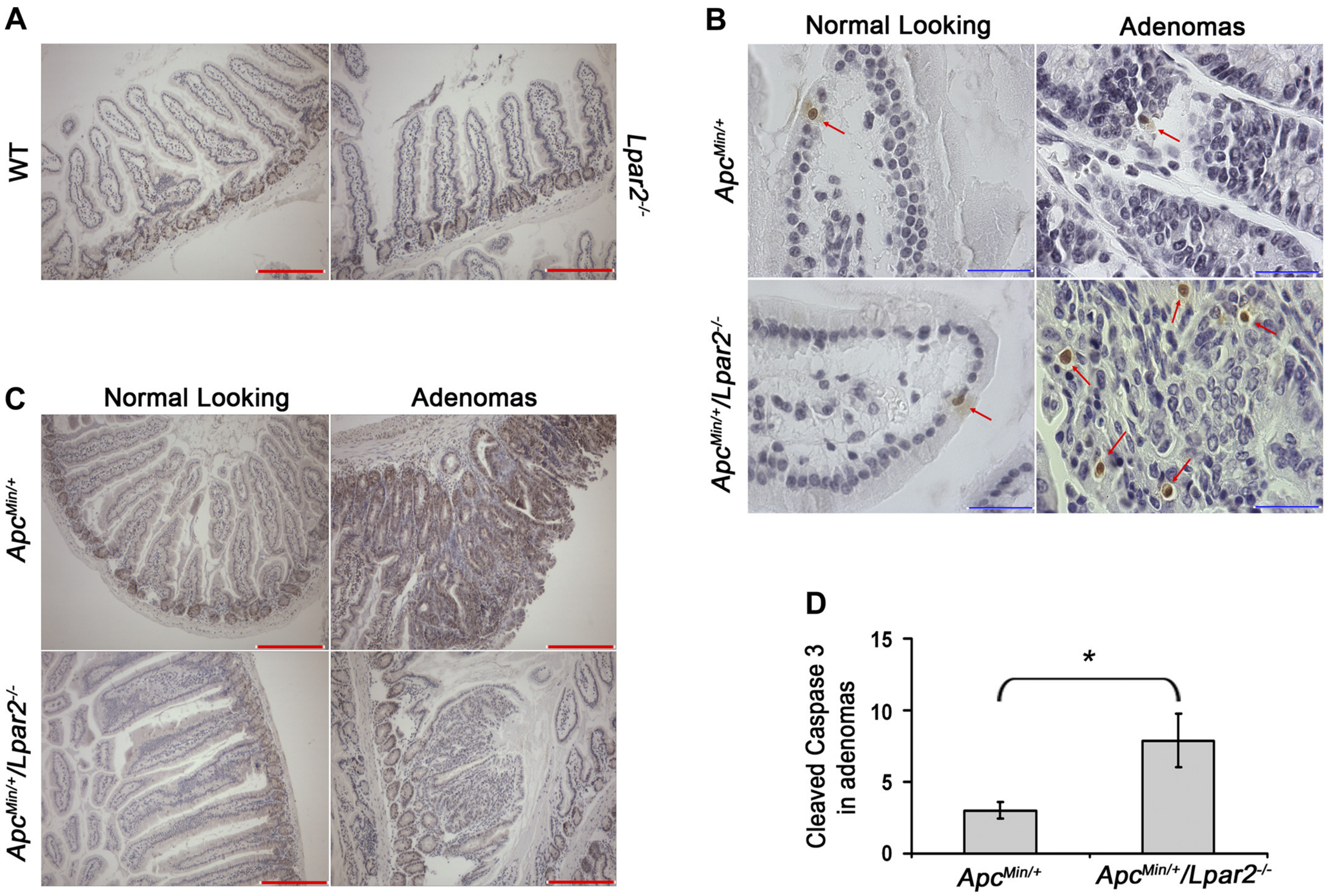

Fig. 3. Cell proliferation and apoptosis in *Apc$^{Min/+}$* and *Apc$^{Min/+}$/Lpar2$^{-/-}$* mice. *A*: intestinal sections from WT and *Lpar2$^{-/-}$* mice were immunolabeled using the antibodies against Ki67. Scale bar: 200 μm. *B*: intestinal sections from *Apc$^{Min/+}$* and *Apc$^{Min/+}$/Lpar2$^{-/-}$* mice were immunolabeled using an antibody against cleaved caspase-3. *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 blue): 30 μm. *C*: intestinal sections from *Apc$^{Min/+}$* and *Apc$^{Min/+}$/Lpar2$^{-/-}$* mice were immunolabeled using an anti-Ki67 antibody. *Left*, intestinal sections of normal-appearing ileum of 21-wk-old mice. *Right*, adenomatous lesions. 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.

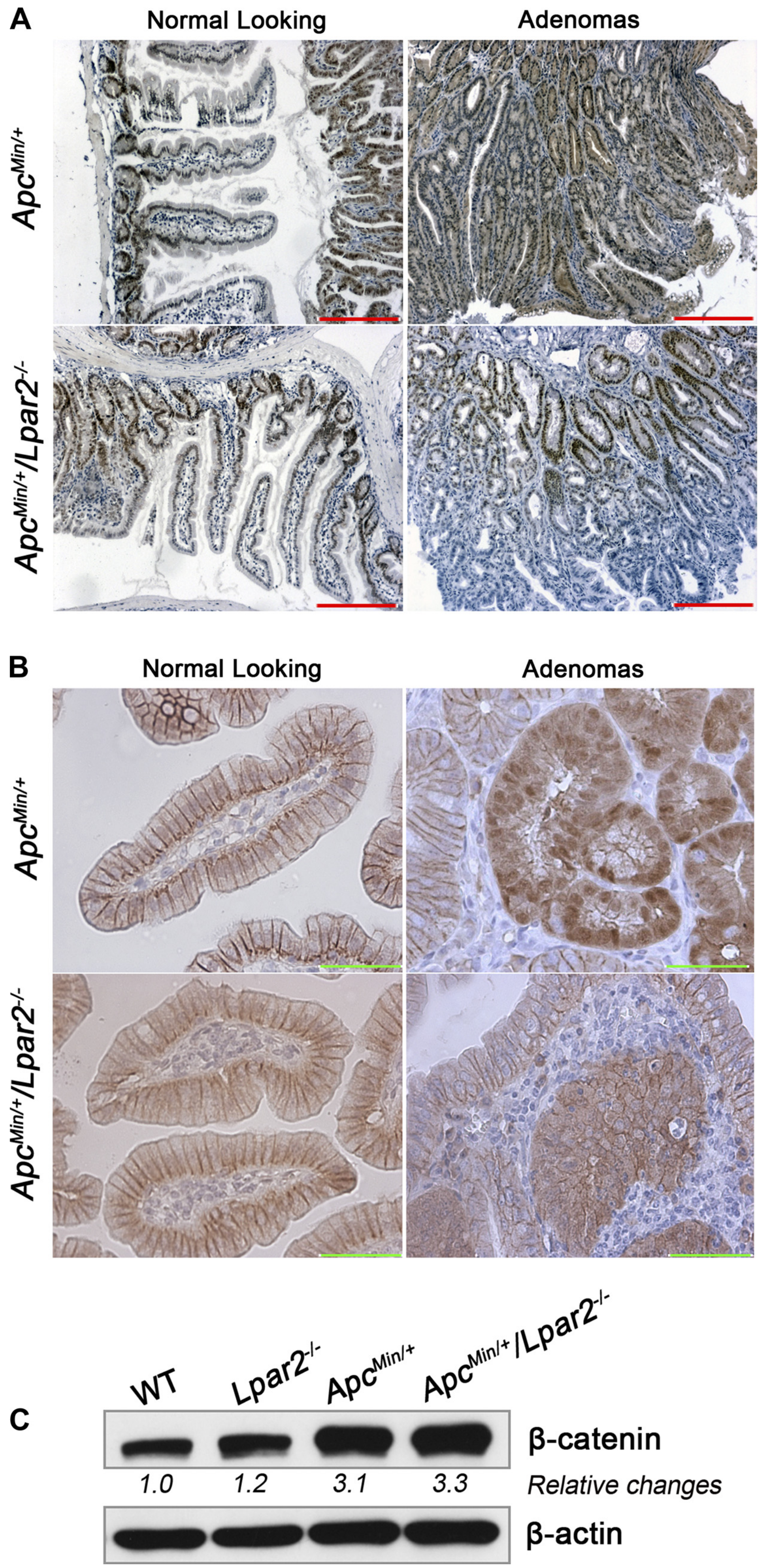

Fig. 4. Krüpple-like factor 5 (KLF5) and β-catenin expression in *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice compared with *Apc*$^{Min/+}$ mice. Intestinal sections of 21-wk-old *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice were stained for KLF5 (*A*) and β-catenin (*B*). *Left*, intestinal sections 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*$^{Min/+}$ and *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ 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 LPA$_2$ expression (data not shown), and, similarly, no significant difference was observed between the healthy intestinal tissues of $Apc^{Min/+}$ and $Apc^{Min/+}/Lpar2^{-/-}$ mice (Fig. 4*A*). Whereas the tumors of $Apc^{Min/+}$ mice showed heightened staining for KLF5 with the staining extending to the surface of tumor, KLF5 staining in the tumors of $Apc^{Min/+}/Lpar2^{-/-}$ 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 LPA$_2$ expression. Immunohistochemical staining of intestinal sections of $Apc^{Min/+}$ mice showed prominent nuclear staining of β-catenin in epithelial cells within adenomatous tumors (Fig. 4*B*). Surprisingly, β-catenin staining in the tumors of $Apc^{Min/+}/Lpar2^{-/-}$ mice was primarily in the cytoplasm, and fewer cells had nuclear β-catenin staining compared with $Apc^{Min/+}$ mice. On the contrary, the total expression level of β-catenin was not significantly altered based on Western blot (Fig. 4*C*), suggesting that the loss of LPA$_2$ 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 LPA$_2$ (data not shown) or before the onset of adenoma in $Apc^{Min/+}$ mice (Fig. 5, *A* and *B*, *left*). On the contrary, the tumors of $Apc^{Min/+}/Lpar2^{-/-}$ mice exhibited reduced levels of cyclin D1 and c-Myc compared with $Apc^{Min/+}$ 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 $Apc^{Min/+}$ mice compared with $Apc^{Min/+}/Lpar2^{-/-}$ mice (Fig. 5*C*).

To ensure that cyclin D1 and c-Myc are downstream targets of LPA$_2$-mediated signaling, LPA$_2$-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 LPA$_2$ expression significantly attenuated the induction of cyclin D1 and c-Myc expression by LPA (Fig. 5*D*). These results indicate that the altered expression of KLF5, β-catenin, cyclin D1, and c-Myc provides the molecular basis

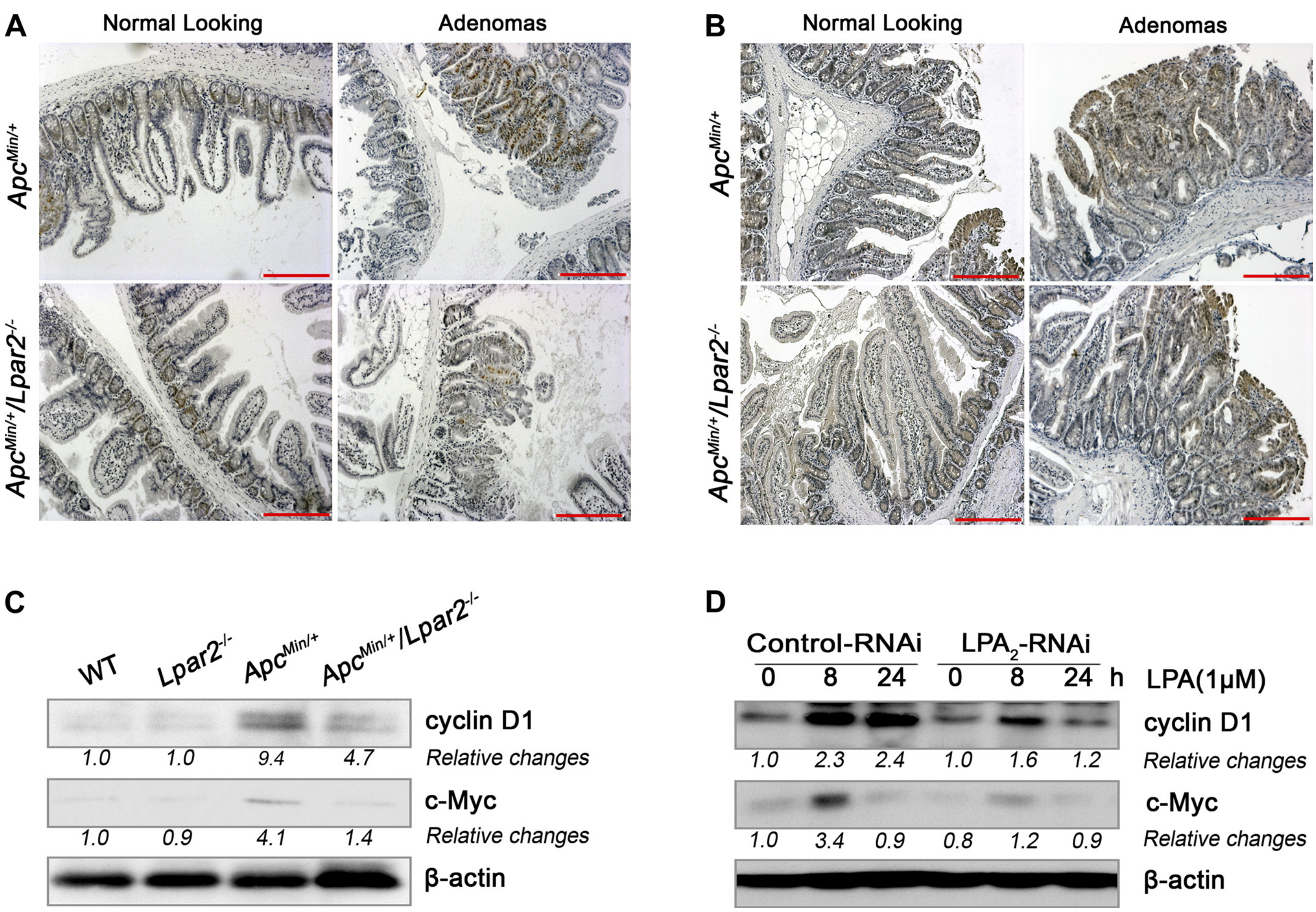

Fig. 5. Reduced levels of cyclin D1 and c-Myc in adenomas of $Apc^{Min/+}/Lpar2^{-/-}$ mice. Immunohistochemical labeling of cyclin D1 (*A*) and c-Myc (*B*) was performed on paraffin-embedded intestinal section of $Apc^{Min/+}$ and $Apc^{Min/+}/Lpar2^{-/-}$ mice. *Left* and *right*, representative images of normal-appearing ileum and adenomatous lesion of 21-wk-old mice, respectively. Scale bar: 200 μm. Representative images from 5 separate experiments are shown. *C*: equal amounts of mouse intestinal lysates were analyzed by Western blotting for the expression levels of cyclin D1 and c-Myc. β-Actin expression was used as a loading control. The relative changes in cyclin D1 and c-Myc expression are indicated. *D*: HCT116 cells transfected with control-RNA interference (RNAi) or LPA$_2$-RNAi were treated with 1 μM LPA for 8 or 24 h, and the expression levels of cyclin D1 and c-Myc were determined by Western immunobotting. β-Actin expression was used as a loading control. Representative results from 4 independent experiments are shown.

for the reduced tumorigenesis in the intestine of *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice.

Apc$^{Min/+}$/*Lpar2*$^{-/-}$ *mice show reduced levels of hypoxia.* 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*$^{-/-}$ mice without a notable difference between the two genotypes (data not shown). Likewise, HIF-1α expression of *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice looked alike. However, in the intestinal adenomas of *Apc*$^{Min/+}$ 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*$^{Min/+}$ mice, which is in contrast to WT and *Lpar2*$^{-/-}$ mice where HIF-1α staining was largely in the cytoplasm with occasional staining in the nuclei (Fig. 6*A*). In *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice, HIF-1α staining was relatively lower than in *Apc*$^{Min/+}$ 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*$^{Min/+}$/*Lpar2*$^{-/-}$ mice was significantly attenuated compared with the intestinal adenomas of *Apc*$^{Min/+}$ 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*$^{Min/+}$ mice compared with *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice when either total intestinal mucosa lysates or individual tumors were compared.

To determine whether HIF-1α activity is altered in *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice relative to *Apc*$^{Min/+}$ 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*$^{Min/+}$/*Lpar2*$^{-/-}$ mice compared with *Apc*$^{Min/+}$ mice (Fig. 6*C*, *top* and *middle*), indicating that HIF-1α activity in tumors is attenuated by the loss of LPA$_2$ function.

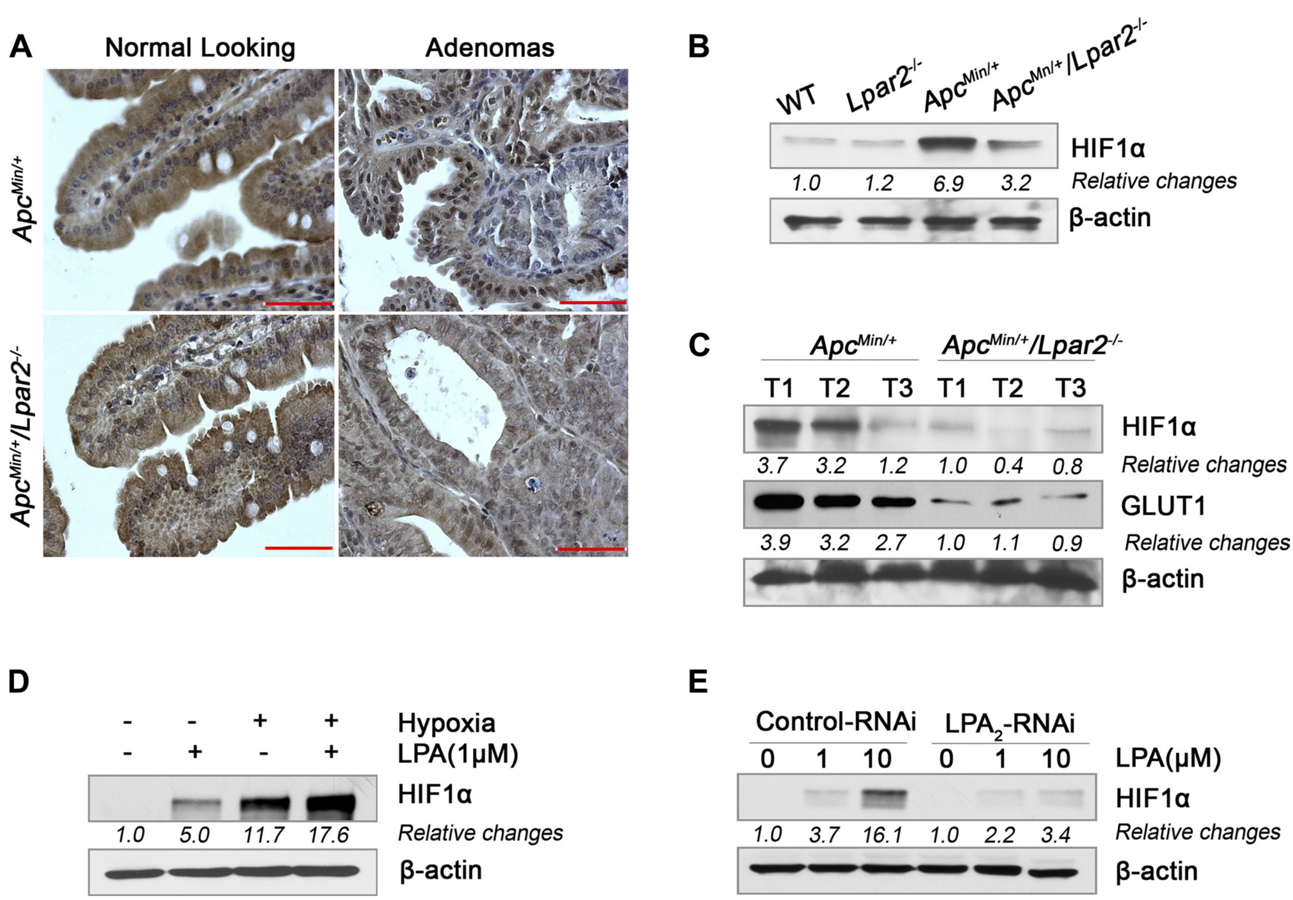

Fig. 6. Reduced hypoxia in *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice. *A*: immunohistochemical labeling of hypoxia-inducible factor-1α (HIF-1α) was performed on paraffin-embedded intestinal sections of *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice. *Left* and *right*, representative images of normal-appearing 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*$^{Min/+}$ or *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ 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 LPA$_2$-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.

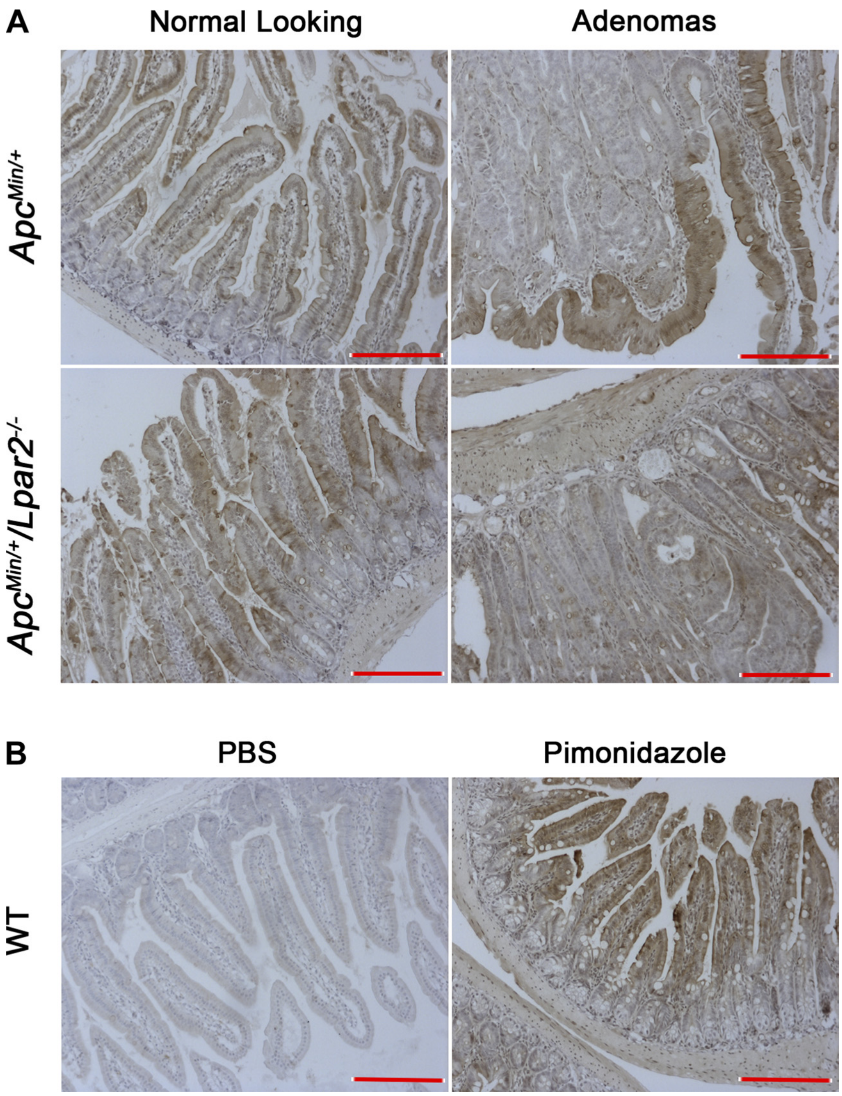

Fig. 7. Altered oxygen gradient in *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice. *A*: mice were treated with pimonidazole (60 mg/kg ip) 3 h before death. Immunohistochemical labeling of antibody against protein adducts of reductively activated 2-nitroimidazoles was performed on paraffin-embedded intestinal sections of *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice. *Left*, intestinal sections of normal-appearing ileum of 21-wk-old mice. *Right*, adenomatous lesions. Scale bar: 200 μm. *B*: intestinal sections of WT mice treated with PBS or pimonidazole were immunolabeled as described in MATERIALS AND METHODS. PBS-treated mice showed no apparent staining with Hypoxyprobe-1 PAb2627 antibodies. Scale bar: 200 μm.

To attain direct evidence that LPA modulates HIF-1α expression in colon cancer cells, HCT116 cells were exposed to LPA. As shown in Fig. 6*C*, 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 LPA_2 expression markedly decreased the activation of HIF-1α expression (Fig. 6*D*), demonstrating that LPA_2 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 *Apc*$^{Min/+}$ 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 LPA_2 attenuates the progression of colon cancer in *Apc*$^{Min/+}$ mice. The absence of LPA_2 did not prevent development of adenomas, but a significant decrease in tumor multiplicity in *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice compared with *Apc*$^{Min/+}$ mice was found. Therefore, the current finding, together with the observation that LPA_2 expression is upregulated in CRC, provides compelling evidence that the LPA-LPA_2 signaling axis is a significant tumor-promoting pathway in the intestinal tract.

Mice lacking LPA_2 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 LPA_2 (7, 23). In the current study, loss of LPA_2 expression in the setting of Apc mutation reduced tumor incidence and size in mouse intestine. The differences in tumor progression between *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/*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 LPA_1 and LPA_2, whereas LPA_2 and LPA_3, but not LPA_1, 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 LPA$_2$ mRNA in human adnocarcinomas and colon cancer cell lines has been demonstrated (41, 51). Nonetheless, it is interesting that the expression level of LPA$_2$ mRNA correlated with increasing sizes of adenomas, raising the possibility that the increased LPA$_2$ 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 *Apc*$^{Min/+}$/*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 LPA$_2$ 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 LPA$_2$ 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 LPA$_2$ (23). In light of these reports, it was unexpected to find that the nuclear translocation of β-catenin was impeded in *Apc*$^{Min/+}$/*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 LPA$_2$-mediated signaling facilitates nuclear translocation of β-catenin. As such, the absence of LPA$_2$ attenuates signaling by EGFR and hence hampers β-catenin activation in *Apc*$^{Min/+}$/*Lpar2*$^{-/-}$ mice. Then, how do we explain that the loss of LPA$_2$ 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 LPA$_2$ 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 LPA$_2$ expression in *Apc*$^{Min/+}$ 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 LPA$_2$ significantly attenuated the initiation and progression of tumor generation via multiple pathways in a mouse model of FAP. Together with the role of LPA$_2$ in colitis-associated colon cancer, our results herein highlight the importance of LPA$_2$ in intestinal tumorigenesis and support for development of LPA$_2$-specific agents as part of therapeutic implementation to prevent and treat CRC.

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DISCLOSURES

The authors have no conflicts of interest.

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