Compensatory Upregulation of LPA2 and Activation of the PI3K-Akt Pathway Prevent LPA5-Dependent Loss of Intestinal Epithelial Cells in Intestinal Organoids

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Abstract: Renewal of the intestinal epithelium is orchestrated by regenerative epithelial proliferation within crypts. Recent studies have shown that lysophosphatidic acid (LPA) can maintain intestinal epithelial renewal in vitro and conditional deletion of Lpar5 (Lpar5iKO) in mice ablates the intestinal epithelium and increases morbidity. In contrast, constitutive Lpar5 deletion (Lpar5cKO) does not cause a defect in intestinal crypt regeneration. In this study, we investigated whether another LPA receptor (LPA R) compensates for constitutive loss of LPA5 function to allow regeneration of intestinal epithelium. In Lpar5cKO intestinal epithelial cells (IECs), Lpar2 was upregulated and blocking LPA2 function reduced proliferation and increased apoptosis of Lpar5cKO IECs. Similar to Lpar5cKO mice, the absence of Lpar2 (Lpar2−/−) resulted in upregulation of Lpar5 in IECs, indicating that LPA2 and LPA5 reciprocally compensate for the loss of each other. Blocking LPA2 in Lpar5cKO enteroids reduced phosphorylation of Akt, indicating that LPA2 maintains the growth of Lpar5cKO enteroids through activation of the PI3K-Akt pathway. The present study provides evidence that loss of an LPAR can be compensated by another LPAR. This ability to compensate needs to be considered in studies aimed to define receptor functions or test the efficacy of a LPAR-targeting drug using genetically engineered animal models.

Keywords: lysophosphatidic acid; LPA5; intestine; epithelial cells; organoid

1. Introduction

The surface of the intestine is lined with a continuous monolayer composed of a variety of epithelial cells, each having specific roles to maintain the critical function of the intestine in nutrient absorption and fluid secretion. In addition, the intestinal epithelium provides a chemical and physical barrier that protects the host from the toxic luminal environment. The intestinal epithelium renews every 3–5 days through a process involving regeneration of new cells by a stable population of intestinal stem cells (ISCs), marked by the leucine-rich repeat-containing G protein-couple receptor 5 (LGR5), residing at the bottom of the crypt [1]. The ISCs give rise to daughter cells that continue to divide and differentiate as they move up towards the surface of the lumen and eventually slough off before being replaced by newly generated cells. ISCs in the crypt base are maintained by multiple signaling molecules secreted by the surrounding stromal environment that regulate the Wnt/b-catenin, Notch, bone morphogenic protein (BMP), epidermal growth factor (EGF), and Hedgehog pathways [2]. In 2009, Sato and co-workers developed a 3-D culture system that indefinitely maintains intestinal stem cells as organoids that form epithelial structures mimicking the crypt–villus structure of the small intestine in a culture dish [3].
In the dynamic environment of the intestine, growth factors have been established to be important mediators of cellular proliferation, differentiation, migration, and survival [4,5]. Lysophosphatidic acid (LPA) is a naturally occurring phospholipid that mediates growth factor-like effects on various cell types and tissues [6]. The effects of LPA are mediated through six distinct LPA receptors (LPARs), termed LPA₁-LPA₆ (gene names Lpar1-Lpar6), which activate major cellular signaling pathways, including the Wnt/b-catenin, phosphatidylinositol-3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways [6]. Each LPAR is coupled to at least one or more of the heterotrimeric Gα proteins, including Gαi/o, Gαq/11, Gα12/13, and Gαs, and LPAR expression varies widely among different tissues and cell types. This variation in LPA receptor expression in part accounts for the dichotomy of LPA-mediated effects observed in different tissues and cell types [7].

The signaling of LPA through LPARs induces diverse effects that are potential mediators during pathological conditions, including rheumatoid arthritis, pulmonary fibrosis, neurodegenerative diseases, and cancer, making LPARs and LPA-mediated signaling potential therapeutic targets. Indeed, research and development of molecules targeting LPARs and the pathways associated with LPA-mediated signaling are underway [8].

To date, knockout (KO) mice have been reported for all six known receptors, and studies of these mice have revealed new pathophysiological roles of LPAR. A targeted deletion of Lpar1 results in neonate lethality with impaired sucking. Surviving Lpar1-deficient (Lpar1⁻/⁻) mice exhibit abnormal phenotypes, such as craniofacial dysmorphism with shorter snouts, wider-spaced eyes, and reduced brain mass [9]. Mice lacking Lpar2 are grossly normal, but an increased incidence of perinatal frontal hematoma is observed in Lpar1/Lpar2 double KO (Lpar1⁻/⁻/Lpar2⁻/⁻) mice compared with Lpar1⁻/⁻ mice, indicating a modest role of LPA₂ in vascular development [9,10]. Lpar3-deficiency results in delayed implantation and embryo crowding and, as a result, Lpar3⁻/⁻ female mice produce reduced litter sizes [11]. Lpar4-deficiency results in defective blood and lymphatic vessel formation during mouse embryogenesis leading to neonatal deaths [12]. A recent study has shown the synergistic role of LPA₄ and LPA₆ in vascular development and that Lpar4/Lpar6 double KO embryos die due to vascular deficiencies with enlarged aortae and poor vascular networks [13].

Mice constitutively lacking Lpar5, Lpar5⁻/⁻, grossly appear normal although decreased pain sensitivity and anxiolytic phenotype are present [14,15]. These mice have a defective intestinal epithelial barrier, which renders them more sensitive to chemical-induced colitis; but otherwise have a normally functioning gastrointestinal system [16]. In contrast, conditional KO of Lpar5 in adult mice results in acute inflammation in the intestinal tract which increases morbidity and mortality of the animals [17]. Loss of Lpar5 in ISCs decreases stem cell functions and reduces the expansion of progenitors [17]. The absence of a gross phenotype by constitutive Lpar5 KO suggests that LPA₅ functions are masked by yet unknown compensatory responses. Here, using IEC-derived enteroids, we show that LPA₂ compensates for the absence of LPA₅ and activates the PI3K-Akt pathway, promoting IEC proliferation and survival.

2. Materials and Methods
2.1. Mice

The generation of Lpar5⁺/⁺ and Lpar5⁺/⁺; Villin-Cre mice was previously described [18]. Lpar5⁺/⁺; RosaCreERT and Lpar5⁺/⁺; AhCre mice were recently described [17].

2.2. Intestinal Crypt Isolation and 3-D Culture of Enteroids

Mouse small intestinal crypts were cultured as previously described [3]. Briefly, the isolated small intestine was cut longitudinally and washed with cold PBS. Crypts were incubated for 1 h at room temperature in Gentle Cell Dissociation Reagent (Stemcell Technologies, Vancouver, BC, Canada) and released from tissue by gentle agitation. Crypts were then passed through a 70 µm cell strainer and the crypt fraction was enriched by centrifugation. Crypts were embedded in Matrigel and cultured in growth media (50%...
Advanced DMEM/F-12, 50% L-WRN, 10% FBS, N-2 media supplement, B-27 supplement, 100 units/mL penicillin, and 0.1 mg/mL streptomycin). Media were replenished every 2–3 days.

2.3. Treatment of Enteroids with Inhibitors

Lpar5<sup>f/f</sup>;Rosa-<sup>Cre</sup>ERT and Lpar5<sup>f/f</sup>;Ah-Cre enteroids were treated with 1 μM 4-hydroxytamoxifen (4OHT) or 1 μM β-naphthoflavone (β-NF), respectively. Lpar5<sup>f/f</sup> and Lpar5<sup>f/f</sup>;Villin-Cre enteroids were treated with an equal volume of sunflower oil. Where indicated, enteroids were treated with 10 μM Ki16425, 10 μM H2L51186303 (Tocris Bioscience, Minneapolis, MN, USA), 10 μM AS2717638 (Fisher Scientific, Hampton, NH, USA), 50 μM LY294002, or 1 μM AG1478 (Sigma-Aldrich, St. Louis, MO, USA). After the formation of enteroids, the media containing inhibitors or carrier were added to enteroids for 48 h.

2.4. Treatment of Mice with LPA<sub>2</sub> Antagonist

Lpar5<sup>f/f</sup> and Lpar5<sup>f/f</sup>;Villin-Cre mice, sex- and age-matched, were given LPA<sub>2</sub> antagonist H2L5186303, 10 mg/kg body weight, by intraperitoneal injection every other day for three times. On day 7, each mouse was administered 5-ethynyl-2′-deoxyuridine (EdU) and euthanized 2 h later.

2.5. Immunofluorescence Staining of Mouse Intestine

After flushing with cold PBS, small intestinal tissues were incubated overnight in 30% sucrose in PBS for cryoprotection. Six mm-thick cryostat sections were prepared and stored at −80 °C until needed. The frozen sections were fixed with ice-cold 100% ethanol and acetone at the ratio of 1:1 for 10 min at −20 °C. EdU-labeled cells were stained using the Click-iT EdU Cell Proliferation kit (Thermo Fisher). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed on intestinal tissues and enteroids using an in situ cell death detection kit (MilliporeSigma, St. Louis, MO, USA) according to the manufacturer’s instruction. Images were taken using a Nikon A1R HD confocal microscope (Nikon Instruments, Melville, NY, USA).

2.6. Immunofluorescence Staining of Enteroids

Enteroids cultured in eight-well chamber slides (Nunc Laboratory-Tek II, MilliporeSigma, Burlington, MA, USA) were fixed with 4% paraformaldehyde for 10 min at room temperature. After fixation, enteroids were permeabilized with 0.2% Triton X-100 for 10 min and blocked in PBS containing 5% goat serum for 1 h at room temperature prior to incubation overnight with rabbit anti-mouse pan-Akt antibodies (4691, Cell Signaling) or rabbit anti-mouse phospho-Akt antibodies (4060, Cell Signaling) at 1:400 dilutions at 4 °C. Slides were then washed with PBS and incubated with Alexa fluor 568 conjugated goat anti-rabbit secondary antibodies (A11036, Invitrogen) at 1:500 dilutions at 4 °C. Slides were then washed with PBS and incubated with Alexa fluor 568 conjugated goat anti-rabbit secondary antibodies (A11036, Invitrogen) at 1:500 dilution for 45 min at room temperature. After washing with PBS, enteroid cells were counterstained with Hoechst (Thermo Fisher Scientific, Waltham, MA, USA) for nuclei. Finally, the slides were viewed under a Nikon A1R HD confocal microscope system with 40× oil objective lens, and images were acquired with the NIS-Elements C Imaging software (Nikon Instruments, Melville, NY, USA).

2.7. Quantitative RT-PCR (qRT-PCR)

Enteroids were harvested to extract total RNA with the RNeasy Mini kit (Qiagen, Hilden, Germany). One μg of total RNA was used for cDNA synthesis using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer’s instruction. Quantitative PCR was performed with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on the Mastercycler Realplex (Eppendorf, Hamburg, Germany). PCR primer sequences are listed in Table 1.
Table 1. Primers used for qRT-PCR.

|    | 5′-3′          |
|----|----------------|
| Lpar1 Forward | CACAGTCAGCAAGCTGGTGATG |
| Lpar1 Reverse | TCTCGAGTATGGGTTCCCTG |
| Lpar2 Forward | TCACAGGTCAATGCGATTG |
| Lpar2 Reverse | AAGGGTGAGTCCATCAGTG |
| Lpar3 Forward | ACGGCTCCCAGGAAAGTAAT |
| Lpar3 Reverse | TACTGACGAGTGGAGGAG |
| Lpar4 Forward | TGACATCGTGAGGATGGTT |
| Lpar4 Reverse | GAAGCTTAAAGCAGTAG |
| Lpar5 Forward | GCTCCAGTGCCCTGACTATC |
| Lpar5 Reverse | GGGAAGTGACAGGGTGAAG |
| Lpar6 Forward | TGACTGGAACACACAGAGCA |
| Lpar6 Reverse | ACTTTGCAAACGGAAATTG |
| ACTB Forward | AGCCATGTACGTAGCCATCC |
| ACTB Reverse | TCTAGCTGTGGTGCTGAAG |

2.8. Statistical Analysis

Statistical analysis was performed by unpaired Student’s t-test, one-way or two-way ANOVA, followed by Tukey post hoc analysis using GraphPad Prism software (Version 9.4, GraphPad Software, San Diego, CA, USA). Results are presented as mean ± SD. A value of p < 0.05 was considered significant.

3. Results

We have recently shown that inducible deletion of Lpar5 in adult mice causes increased crypt epithelial cell death that dysregulates the intestinal epithelial renewal [17]. In contrast, constitutive deletion of Lpar5 does not cause a gross change in the intestine although increased intestinal epithelial permeability is noted [14,18]. These differences are depicted in Figure 1A which compare the growth of intestinal epithelial cell (IEC)-derived enteroids from inducible Lpar5 knockout (Lpar5\textsuperscript{iKO}) and constitutive KO (Lpar5\textsuperscript{cKO}) mice. Mouse intestinal crypt cells seeded in Matrigel in the presence of growth factors, including R-spondin, EGF and Noggin, and serum formed organoids or enteroids with crypt-like extensions as previously demonstrated (Figure 1A, Lpar5\textsuperscript{f/f}) [3]. We have recently shown that Lpar5 expression is almost completely depleted in Lpar5\textsuperscript{f/f};Rosa-Cre\textsuperscript{ERT} or Lpar5\textsuperscript{f/f};Ah-Cre enteroids by treating them with 4OHT or β-NF, respectively [17]. Deletion of Lpar5 resulted in IEC apoptosis and the enteroids lost their structural integrity (Figure 1A, Lpar5\textsuperscript{f/f};Rosa-Cre\textsuperscript{ERT} or Lpar5\textsuperscript{f/f};Ah-Cre). In comparison, the growth of Lpar5\textsuperscript{f/f};Villin-Cre enteroids that constitutively lack Lpar5 expression in IECs [18] was indistinguishable from wild-type (WT; Lpar5\textsuperscript{f/f}) enteroids. This data correlates with what was shown in vivo in the intestinal epithelium of Lpar5\textsuperscript{f/f};Villin-Cre mice [18]. Of note, we have shown previously that 4OHT or β-NF alone has no effect on the growth of WT enteroids [17].
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Figure 1. Constitutive deletion of Lpar5 is associated with growth of enteroids and upregulation of Lpar2 and Lpar3. (A) Enteroids were derived from control Lpar5<sup>f/f</sup>, mice with inducible deletion of Lpar5 (Lpar5<sup>Cre</sup>;<Rosa-Cre<sup>ERT</sup>), Lpar5<sup>f/f</sup>;Ah-Cre), and mice with constitutive, intestinal-specific deletion of Lpar5 (Lpar5<sup>f/f</sup>;Villin-Cre). Lpar5<sup>f/f</sup>;Rosa-Cre<sup>ERT</sup> and Lpar5<sup>f/f</sup>;Ah-Cre were treated with 4OHT or β- NF, respectively, on Day 0 (D0) to delete Lpar5 expression. Images were then taken on D0 and DScale bar = 50 μm. Lpar1–6 mRNA expression levels in Lpar5<sup>f/f</sup> enteroids (B) and Lpar5<sup>f/f</sup>;Villin-Cre entoroids (C) were determined and normalized to β-actin mRNA levels. Results representing three independent experiments with quadruplicates for each sample are shown. (D) Lpar1–6 expression levels in Lpar5<sup>f/f</sup>;Villin-Cre enteroids (KO) were expressed relative to WT enteroids. (E) Lpar1–6 mRNA expression in intestinal mucosal tissues from Lpar5<sup>f/f</sup>;Villin-Cre (KO) relative to WT mice is shown.

In light of both the striking differences between these enteroids and previous studies demonstrating overlaps in signaling by different LPARs, we hypothesized that one or more LPARs might compensate for the absence of Lpar5 in Lpar5<sup>f/f</sup>;Villin-Cre enteroids, maintaining the survival of IECs. To examine this, Lpar mRNA expression levels were determined in Lpar5<sup>f/f</sup> and Lpar5<sup>f/f</sup>;Villin-Cre enteroids. Since the annealing and polymerization efficacies of the primer set for each Lpar are not normalized, we quantified Lpar mRNA expression levels relative to β-actin mRNA expression levels. Consistent with our previous studies, Lpar1, Lpar5, and Lpar6 mRNA were more abundantly expressed in WT enteroids compared to Lpar2, Lpar3, and Lpar4 mRNA (Figure 1B). In comparison, Lpar2 mRNA expression was markedly elevated in Lpar5<sup>f/f</sup>;Villin-Cre enteroids (Figure 1C). In addition, Lpar3 mRNA expression was tripled in Lpar5<sup>f/f</sup>;Villin-Cre enteroids compared with WT entoroids (Figure 1D). Expression levels of Lpar1, Lpar4, and Lpar6 were not significantly

mRNA expression was tripled in Lpar5<sup>f/f</sup>;Villin-Cre enteroids (Figure 1D). Expression levels of Lpar1, Lpar4, and Lpar6 were not significantly
changed. Similarly, comparing mRNA levels in mouse intestinal lysates corroborated increased Lpar2 and Lpar3 mRNA expression in Lpar5\(^{f/f}\);Villin-Cre mice compared with WT mice (Figure 1E).

Given the increased Lpar2 and Lpar3 mRNA expression in Lpar5\(^{f/f}\);Villin-Cre enteroids, we next determined whether LPA\(_2\) or LPA\(_3\) contributes to the growth of Lpar5\(^{f/f}\);Villin-Cre enteroids. Inhibition of LPA\(_2\) by using LPA\(_2\) antagonist H2L5186303 blocked enteroid growth (Figure 2A,B) and compromised their survival (Figure 2C) [19,20]. By contrast, Ki16425, which inhibits both LPA\(_1\) and LPA\(_3\) [21], did not have a significant effect on enteroid growth or survival (Figure 2A–C). These results were reinforced by confocal immunofluorescence (IF) microscopic analysis, which showed that inhibition of LPA\(_2\) decreased cell proliferation (EdU), while increasing apoptosis assessed by TUNEL staining (Figure 2D). Despite increased Lpar3 mRNA expression levels, LPA\(_1\)/LPA\(_3\) antagonist Ki16425 did not alter the extent of cell proliferation or apoptosis. These results indicated that LPA\(_2\) compensates for the loss of LPA\(_3\) function to promote IEC proliferation and prevent apoptosis.

**Figure 2.** Inhibition of LPA\(_2\) in Lpar5\(^{f/f}\);Villin-Cre enteroids results in reduced proliferation and increased apoptosis of IECs. (A) Lpar5\(^{f/f}\);Villin-Cre enteroids were treated with 10 µM LPA\(_2\) inhibitor H2L5186303 (H2L5) or LPA\(_1\)/LPA\(_3\) inhibitor Ki16425 (Ki16) on day 0 (D0). Representative images taken on D0 and D2 are shown. n = Bar = 50 µm. (B) The growth of enteroids was quantified by determining the surface area of enteroids using ImageJ. All data are presented as mean ± SD. *p < 0.05, ****p < 0.0001 by two-way ANOVA with Tukey’s multiple comparison test. n = 10. (C) Number of viable enteroids counted on D0 and D2 and expressed as percentage on D0. n = 4 wells per condition. ****p < 0.0001, ns = not significant. (D) Lpar5\(^{f/f}\);Villin-Cre enteroids were treated with H2L5 or Ki. Representative IF images of DAPI (blue), EdU (green), and TUNEL (red) taken on D2 are shown. Bar = 50 µm. (E) Numbers of Edu+ (upper panel) and TUNEL+ (lower panel) cells per enteroid were quantified. n = 10. ****p < 0.0001 and ns = not significant compared with control (Con) by one-way ANOVA with Tukey’s multiple comparison test.
To confirm the role of LPA₂ on IEC proliferation and survival in the absence of LPA₅, we administered LPA₂ inhibitor H2L5186303 to Lpar5⁻/⁻;Villin-Cre mice at 10 mg/kg concentrations by intraperitoneal injection every other day and mice were euthanized on day 5. Similar to the findings in enteroids, inhibition of LPA₂ significantly decreased the number of proliferating cells in Lpar5⁻/⁻;Villin-Cre intestinal crypts while increasing apoptosis (Figure 3A,B). Interestingly, H2L5186303 led to a small but statistically significant decrease in the number of proliferating cells in control Lpar5⁻/⁻ mouse crypts, suggesting that LPA₂ may play a small role in the regulation of IEC proliferation under basal conditions.

Figure 3. LPA₂ inhibitor significantly alters IEC proliferation and survival in Lpar5⁻/⁻;Villin-Cre mouse intestine. Representative images of EdU (green) (A) and TUNEL (red) (B) fluorescence staining in the intestinal sections of Lpar5⁻/⁻ and Lpar5⁻/⁻;Villin-Cre mice treated with DMSO (Con) or H2L5186303 (H2L5). Bar = 100 μm. Quantification of EdU+ cells per crypt and TUNEL+ per crypt are shown below. n = 60. * p < 0.05, **** p < 0.0001, ns = not significant by unpaired, two-tailed t-test.

To ascertain the role of LPA₂ in promoting IEC proliferation and survival, we subjected WT enteroids to LPA₂ inhibitor H2L5186303. Inhibition of LPA₂ attenuated the growth of enteroids along with changes in cell proliferation and apoptosis (Figure 4A–E, H2L5 vs. Con), corroborating the in vivo findings. Consistent with our recent study that conditional deletion of Lpar5 or LPA₅ antagonist TC LPA₅ 4 blocks the growth of WT enteroids [17], LPA₅ antagonist AS2717638 suppressed the growth of WT enteroids (Figure 4A,B, AS27 vs. Con) [22]. The effects of LPA₂ and LPA₅ antagonists were corroborated by IF staining of cell proliferation and apoptosis (Figure 4C–E). Simultaneous antagonism of LPA₂ and LPA₅ further suppressed enteroid growth compared to LPA₂ inhibition alone (Figure 4B, H2L5+AS27 vs. H2L5), but no statistical difference was observed between enteroids treated with AS2717638 alone and those co-treated with H2L5186303+AS2717638.
Lpar5 whether a similar compensatory mechanism prevails in the Lpar2 was virtually indistinguishable from WT enteroids (Figure 5A). Hence, we investigated inhibited the growth of Lpar2 inhibitor Ki16425 or LPA enteroids compared with WT enteroids (Figure 5B). To assess whether LPA by RT-PCR revealed elevated expression levels of Lpar5 in particular, given that LPA basal conditions [10,23]. Consistently, the growth of enteroids derived from Lpar2−/− mice and the intestinal tract appears normal under apparent phenotypic abnormality in mice and the intestinal tract appears normal under basal conditions [10,23]. Consistently, the growth of enteroids derived from Lpar2−/− mice was virtually indistinguishable from WT enteroids (Figure 5A). Hence, we investigated whether a similar compensatory mechanism prevails in the Lpar2−/− mouse intestine. In particular, given that LPA2 can compensate for the absence of LPA5, we questioned whether Lpar5 expression is elevated in Lpar2−/− IECs. Analysis of Lpar mRNA expression by RT-PCR revealed elevated expression levels of Lpar3 and Lpar5 mRNA in Lpar2−/− enteroids compared with WT enteroids (Figure 5B). To assess whether LPA3 or LPA5 is functionally important, Lpar2−/− enteroids were cultured in the presence of LPA3 inhibitor Ki16425 or LPA5 inhibitor AS2717638. We found that AS2717638 significantly inhibited the growth of Lpar2−/− enteroids (Figure 5C,D, Con vs. AS27), but Ki16425 also

Figure 4. The growth of WT enteroids is suppressed by inhibition of LPA2 and LPA5. (A) Representative images of Lpar5fl/fl enteroids treated with LPA2 inhibitor H2L5186303 (H2L5), LPA1/LPA3 inhibitor Ki16425 (Ki16), LPA3 inhibitor AS2717638 (AS27), or H2L5+AS27 are shown. Data are representative of 3 experiments. Scale bar = 50 μm. (B) The growth of Lpar5fl/fl enteroids was quantified by determining the area of enteroids using ImageJ. All data are presented as mean ± SD. n = 10. **p < 0.01, ***p < 0.001, ns = not significant by two-way ANOVA with Tukey’s multiple comparison test. (C) Representative images of EdU and TUNEL staining in Lpar5fl/fl enteroids treated with inhibitors are shown. Representative of three independent experiments. Bar = 50 μm. Quantification of EdU+ (D) and TUNEL+ ****p < 0.0001, ns = not significant compared to Con. (E) enteroid. n = 10. **p < 0.01, ****p < 0.0001, ns = not significant compared to Con. #p < 0.0001.

Similar to Lpar5KO mice, constitutive knockout of Lpar2 (Lpar2−/−) does not cause apparent phenotypic abnormality in mice and the intestinal tract appears normal under basal conditions [10,23]. Consistently, the growth of enteroids derived from Lpar2−/− mice was virtually indistinguishable from WT enteroids (Figure 5A). Hence, we investigated whether a similar compensatory mechanism prevails in the Lpar2−/− mouse intestine. In particular, given that LPA2 can compensate for the absence of LPA5, we questioned whether Lpar5 expression is elevated in Lpar2−/− IECs. Analysis of Lpar mRNA expression by RT-PCR revealed elevated expression levels of Lpar3 and Lpar5 mRNA in Lpar2−/− enteroids compared with WT enteroids (Figure 5B). To assess whether LPA3 or LPA5 is functionally important, Lpar2−/− enteroids were cultured in the presence of LPA3 inhibitor Ki16425 or LPA5 inhibitor AS2717638. We found that AS2717638 significantly inhibited the growth of Lpar2−/− enteroids (Figure 5C,D, Con vs. AS27), but Ki16425 also
exerted a small but statistically significant effect on Lpar2−/− enteroids compared with controls (Figure 5D, Con vs. Ki16). As expected, LPA2 antagonist H2L5186303, which was used as a control, had no effect (Con vs. H2L5). These results were corroborated by IF microscopic analysis of cell proliferation (Figure 5E, EdU, and Figure 5F) and apoptosis (Figure 5E, TUNEL, and Figure 5G). AS2717638 consistently imposed a greater effect on intestinal crypt proliferation. **p < 0.01, ****p < 0.0001 compared to WT.

**Figure 5.** Increased Lpar5 compensates for the loss of Lpar2. (A) Representative images of enteroids derived from WT and Lpar2−/− mice are shown. Bar = 50 μm. (B) Relative expression levels of Lpar1-6 expression in Lpar2−/− compared with WT enteroids. ***p < 0.001, ****p < 0.0001 compared to WT. n = 4. (C) Representative images of Lpar2−/− enteroids treated with inhibitors are shown. (D) The number of viable enteroids in the presence of LPAR inhibitors was quantified by determining the average surface area of enteroids using ImageJ (version 1.8, National Institutes of Health, Bethesda, MD, USA). **p < 0.01, ****p < 0.0001, ns = not significant by one-way ANOVA with Tukey’s multiple comparison test. (E) The number of viable enteroids were counted on D0 and D2 and presented as % of D0. ****p < 0.0001, ns = not significant by two-way ANOVA with Tukey’s multiple comparison test. (F) Representative images of EdU (green) and TUNEL (red) staining of Lpar2−/− enteroids are shown. DAPI = blue. Bar = 50 μm. Quantification of EdU+ (G) and TUNEL+ cell numbers **p < 0.01, ****p < 0.0001, and ns = not significant compared to control. #p < 0.01. (H) Representative of three independent experiments. **p < 0.01, ****p < 0.0001, and ns = not significant compared to control.
We next investigated the mechanism of LPA_2-dependent growth of Lpar_5^{−/−}/Villin-Cre enteroids. Previous studies have shown that LPA_2 facilitates human colon Caco-2 cell survival via activation of MAPK [24, 25]. Moreover, a recent study has shown that LPA can substitute EGF to support the growth of mouse enteroids [26]. However, the stem cell culture media contain EGF which should activate EGF receptors independent of the Lpar expression status. This assumption was supported by the observation that inhibition of EGFR by AG1478 prevented the growth of Lpar_5^{−/−} enteroids (Figure 6). In addition to the MAPK pathway, LPA activates the PI3K-Akt pathway, which mediates proliferative signals in the intestine [25, 27, 28]. Therefore, we investigated the role of the PI3K-Akt pathway in sustaining proliferation and survival of Lpar_5^{−/−} IECs. We asserted the importance of PI3K in IEC proliferation by demonstrating the inhibitory effect of PI3K inhibitor LY294002 and LPA_2 antagonist H2L5186303 similarly blocked the growth of Lpar_5^{−/−} enteroids (Figure 7A, B). Confocal IF images show the presence of phosphorylated Akt (p-Akt) in the crypt-like domains of Lpar_5^{−/−} enteroids, which was suppressed by PI3K inhibition (Figure 7C, D, LY). Importantly, a similar decrease in p-Akt levels was observed in enteroids treated with LPA_2 antagonist H2L5186303 (Figure 7C, H2L5). Co-treatment of the enteroids with H2L5186303 and LY294002 did not yield an additive effect on enteroid growth (Figure 7A) or p-Akt (Figure 7C, D) compared to enteroids treated with either inhibitor. Taken together, these data suggest that LPA_2 regulates the PI3K-Akt pathway, which is critical for the growth of Lpar_5^{−/−} enteroids. We next questioned whether LPA_5-mediated effect in Lpar_2^{−/−} enteroids is also dependent on the PI3K-Akt pathway. To address this question, Lpar_2^{−/−} enteroids were cultured in the presence of LPA_5 antagonist AS2717638, PI3K inhibitor LY294002, or both together. The growth of Lpar_2^{−/−} enteroids was halted by AS2717638, as we demonstrated in Figure 7E. Importantly, inhibition of PI3K by LY294002 effectively obliterated growth of Lpar_2^{−/−} enteroids (Figure 7E), demonstrating that the PI3K-Akt pathway is involved in the compensatory rescue of Lpar_2^{−/−} enteroids.

**Figure 6.** Inhibition of EGFR prevents Lpar_5^{−/−} enteroid growth. Lpar_5^{−/−} enteroids were cultured in the presence of LPA_2 inhibitor H2L5186303 (H2L5), or EGF inhibitor AG1478 (AG). Representative images of enteroids on D0 and D2 (upper panel) and average surface area of enteroids (lower panel) are shown. Bar = 50 μm. **** p < 0.0001, and ns = not significant by two-way ANOVA with Tukey’s multiple comparison test.
Figure 7. LPA₂ promotes the growth of Lpar5⁻/⁻ enteroids via activation of the PI3K-Akt pathway.

(A) Lpar5⁻/⁻ (Lpar5⁰/⁰;Villin-Cre) enteroids were cultured in the presence of LPA₂ inhibitor H2L5186303 (H2L5), PI3K inhibitor LY294002 (LY), or both. Representative images of enteroids taken on D0 and D2 are shown. Bar = 50 μm. (B) Growth of enteroids was quantified by determining the average surface area of enteroids. *p < 0.05, ****p < 0.0001, and ns = not significant by two-way ANOVA with Tukey’s multiple comparison test. (C) Representative immunofluorescence images for p-Akt (upper
panels) and Akt (lower panels) are shown. DAPI = blue. Images are representative of three independent experiments. Bar = 50 µm. (D) Ratios of p-Akt to total Akt fluorescence intensity (mean ± SD) are shown. * p < 0.0001 and ns = not significant by one-way ANOVA with Tukey’s multiple comparison test. (E) Growth of Lpar2−/− enteroids in the presence of LPA5 inhibitor AS2717638 (AS27), PI3K inhibitor LY294002 (LY), or both were determined and average surface areas of enteroids (mean ± SD) under each condition are shown. * p < 0.05, **** p < 0.0001, and ns = not significant by two-way ANOVA with Tukey’s multiple comparison test.

4. Discussion

The relative absence of phenotypic aberration by germline LPAR deletion suggests that either LPAR functions do not significantly contribute to the fetal development or other cellular processes compensate for the absence of the LPAR. The possibility of compensatory adaptation by another LPAR(s) is probable based on the significant overlap in the cellular signaling pathways and functions of LPARs. This study is aimed at exploring the presence of a possible compensatory mechanism in Lpar5cKO mice. We show that upregulation of LPA2 compensates for the loss of LPA5 function to maintain epithelial cell proliferation in the intestine. Our study demonstrates that LPA2-mediated compensatory effect is dependent on the PI3K-Akt pathway.

To identify the LPA receptor potentially compensating for the absence of LPA5, we utilized ex vivo cultures of intestinal epithelial stem cells from mice. The intestinal stem cells expressing LGR5 form crypt-like structures or enteroids in laminin-rich Matrigel supplemented with a cocktail of growth factors, including Noggin, R-spondin, and EGF [1]. Recently, we have demonstrated that inducible deletion of Lpar5 compromises the intestinal crypt regeneration in vivo and impedes the formation of 3-D intestinal enteroids in vitro, demonstrating the autonomous role of LPA5 in the self-renewal of the intestinal epithelium [17]. In contrast, the intestinal mucosal architecture of Lpar5cKO mice appeared normal and the growth patterns of Lpar5cKO and WT enteroids were alike, suggesting a compensatory rescue of the Lpar5 deficiency. Comparison of Lpar mRNA levels showed increased expression levels of Lpar2 and Lpar3 in Lpar5cKO enteroids, and pharmacological inhibition of LPA2 prevented IEC proliferation and survival. Although Lpar3 mRNA expression was elevated in Lpar5cKO mouse enteroids, LPA1/LPA3 antagonist Ki16425 did not have a significant effect on enteroids. These results are corroborated by a recent study that Ki16425 did not have a significant effect on WT enteroids [26]. However, we cannot rule out the possibility of ineffective delivery of KiMoreover, a concern over the acute pharmacological treatment vs. the long-term effects of gene KO could not be addressed in the current study. The role of LPA2 in protection of IECs has been demonstrated by previous studies where activation of LPA2 provides protection against radiation-induced intestinal damage [29–31]. The presence of Lpar2 in ISC is not clear but a comparison of Lpar2 mRNA along the crypt–villus axis of the mouse intestine has shown an increased abundance of Lpar2 mRNA in the crypts compared to villi [32]. The expression of Lpar2 in the crypts correlates with its function in epithelial regeneration. Moreover, pretreating mouse enteroids with a LPA2 agonist was shown to protect LGR5+ ISC from irradiation [33]. Our current study confirmed the effect of LPA2 inhibition in vivo where a significant increase in IEC death along with decreased IEC proliferation in the intestinal crypts of Lpar5f/f;Villin-Cre mice were observed. Surprisingly, a small decrease in IEC proliferation by LPA2 antagonist H2L5226501 was noted in control Lpar5f/f mice, which is contrary to our previous study that no basal change in IEC proliferation was observed in Lpar2−/− mice [23]. However, we cannot rule out the possibility that the effect of H2L5226501 is caused by a lack of specificity for LPA2 mouse strain with inducible Lpar2 KO is needed to fully explore the role of LPA2 in epithelial regeneration in the intestine.

Lpar1 mRNA is highly expressed in IECs, and we have observed reduced IEC proliferation in Lpar1−/− mice [34,35]. However, Lpar1 expression was not altered in Lpar5cKO enteroids compared with WT controls, and LPA1/LPA3 antagonist Ki16425 did not have a significant effect on enteroids. The difference in IEC proliferation between Lpar1−/−
mice and Ki16425 treatment of enteroids can be attributed to a probable compensatory adaptation in Lpar1−/− mice. Similarly, previous studies comparing genetic deletion of Lpar1 and pharmacological blockage of LPA1 function by Ki16425 on mouse behavior and brain functions found that the antagonist mimicked some, but not all, of the effects of Lpar1 deletion [36,37].

Because LPA2 compensates for the absence of LPA5, we questioned whether Lpar2−/− enteroids were protected by a similar compensatory process. We found increased Lpar5 mRNA expression in Lpar2−/− enteroids and the blockade of LPA5 function by AS2717638 suppressed the growth of Lpar2−/− enteroids. In addition, Lpar3 mRNA levels were doubled in Lpar2−/− enteroids relative to WT controls and LPA1/LPA3 antagonist Ki16425 appeared to have a moderate effect on Lpar2−/− enteroid growth. The effect of Ki16425 was surprising given the lack of effect on Lpar5−/− enteroids. However, one caveat of the current study is the assessment of LPAR expression by mRNA levels which do not always correlate with protein expression. We could not reliably determine LPAR protein expression due to the lack of reliable antibodies against LPAR. Nevertheless, the results in this study collectively demonstrate that a germline loss-of-function mutation of a LPAR is likely to be compensated by another LPAR. Similar to the current study, upregulation of related genes following a gene knockout has been observed previously [38]. For example, the loss of the ribosomal Rpl22 gene results in a subtle phenotypic change due to a compensatory increase in Rpl22-like1 gene expression [39]. Germline PKM2-null mice (Pkm2−/−) are viable and fertile, despite PKM2 being the primary isoform expressed in most wild-type adult tissues, due to compensatory expression of related PKM1 [40].

The EGF signaling pathway is essential for the proliferation of IECs [41], and intestinal enteroids cannot maintain their growth without EGF [3]. The transactivation of EGFR by GPCR, including LPAR, is well-established [42,43]. A recent study demonstrated the ability of LPA to substitute EGF to promote the development and growth of intestinal enteroids [26]. Consistent with the observation that LPA promotes enteroid growth through transactivation of EGFR, inhibition of LPA1 impeded enteroid growth in a media containing LPA but not EGF [26]. However, defective EGFR activation cannot account for the inability of Lpar5-deficient enteroids to grow since EGF was present in all experiments performed in the current study. Indeed, inhibition of EGFR or MEK prevented Lpar5-deficient enteroid growth, demonstrating that the EGF signaling was intact. LPA, present in serum in micromolar concentrations, activates multiple signaling pathways, including MEK-ERK, PI3K-Akt, phospholipase C, and p38 MAPK pathways [44,45]. We focused on the PI3K-Akt pathway based on previous studies demonstrating the critical role of the PI3K-Akt pathway in promoting IEC proliferation [27]. We found that chemical inhibition of PI3K suppressed the growth of Lpar5−/− enteroids, demonstrating the critical role of the PI3K-Akt pathway in the stimulation of IEC proliferation. Importantly, inhibition of LPA2 similarly attenuated activation of Akt, and blocking LPA2 and PI3K functions did not have an additive effect, suggesting that LPA2− and PI3K-dependent signaling are vertically integrated. The role of the PI3K-Akt pathway in supporting compensatory growth was recapitulated in Lpar2−/− enteroids where inhibition of LPA5 or PI3K blocked the growth of Lpar2−/− enteroids.

Despite evidence associating LPAR to experimental models of colitis [16,35,46], genome-wide association studies have not linked mutations in a Lpar gene to disorders of the gastrointestinal tract in humans. One exception to that is the identification of a single nucleotide polymorphism in GPCR GPR35 (rs4676410) among patients with inflammatory bowel disease (IBD) [47,48]. LPA is one of several endogenous ligands of GPR35, associating aberrant LPA-mediated signaling to the pathogenesis of IBD [49]. If the compensatory responses of LPAR-deficiency exist in humans, it is possible that defects associated with in-born mutations in a Lpar gene could be masked partially or fully by compensatory upregulation of another LPAR. Another possibility is that the extent of penetration of variant Lpar allele is not deep enough to provide sufficient statistical power in polygenic, multifaceted chronic inflammatory diseases. Further studies are needed to investigate whether a variant Lpar allele is present in a subset of IBD patients.
Here, we demonstrated that the constitutive loss of a LPAR is compensated by another LPAR. Specifically, we showed that LPA₅ loss of function in intestinal crypt epithelium is compensated by upregulation of LPA₂ activity, which activates the PI3K-Akt pathway. LPAR expression varies among different cell types and tissues so that there is the possibility that the compensatory adaptation in other cells, tissues, or organs might be modulated by a different member of the LPAR family. Nonetheless, this ability to compensate loss of a LPAR through upregulation of another LPAR needs to be considered in future studies that are aimed to define receptor functions or test the efficacy of a LPAR-targeting drug using genetically engineered animal models.

**Author Contributions:** Z.L. performed all experiments, analyzed data, and drafted the manuscript. C.C.Y. analyzed data, supervised the study, obtained funding, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the grant from the National Institutes of Health (R01DK116799) and the Veterans Administration Merit Award (I01BX004459). Confocal microscopic analyses were supported in part by the Integrated Cellular Imaging Shared Resources of Winship Cancer Institute of Emory University and NIH/NCI under award P30CA138292.

**Institutional Review Board Statement:** All animal experiments were conducted under approval by the Institutional Animal Care and Use Committee of Emory University (PROTO201700753) and in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Acknowledgments:** We thank Beth McConnell for proofreading the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

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