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Glutamine and alanyl-glutamine promote crypt expansion and mTOR signaling in murine enteroids

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Glutamine and alanyl-glutamine promote crypt expansion and mTOR signaling in murine enteroids. Am J Physiol Gastrointest Liver Physiol 308: G831–G839, 2015. First published March 19, 2015; doi:10.1152/ajpgi.00422.2014.—L-Glutamine (Gln) is a key metabolic fuel for intestinal epithelial cell proliferation and survival and may be conditionally essential for gut homeostasis during catabolic states. We show that L-alanyl-L-glutamine (Ala-Gln), a stable Gln dipeptide, protects mice against jejunal crypt depletion in the setting of dietary protein and fat deficiency. Separately, we show that murine crypt cultures (enteroids) derived from the jejunum require Gln or Ala-Gln for maximal expansion. Once expanded, enteroids deprived of Gln display a gradual atrophy of cryptlike domains, with decreased epithelial proliferation, but stable proportions of Paneth and goblet cell differentiation. At 24 h, depletion of enteroid medium with Gln selectively activates mammalian target of rapamycin (mTOR) signaling pathways in murine enteroids, rescues proliferation, and promotes crypt regeneration. Gln deprivation beyond 48 h leads to destabilization of enteroids but persistence of EGFP-Lgr5-positive intestinal stem cells with the capacity to regenerate enteroids upon Gln rescue. Collectively, our findings indicate that Gln deprivation induces a reversible quiescence of intestinal stem cells and provides new insights into nutritional regulation of intestinal epithelial homeostasis.

intestinal organoids; L-glutamine; L-alanyl-L-glutamine; ERK; mammalian target of rapamycin

IMPORTANT QUESTIONS regarding the maintenance of gastrointestinal (GI) epithelial homeostasis by the intestinal stem cells (ISCs) residing within the crypts of Lieberkühn are 1) how decisions about cellular growth, differentiation, and death are made and 2) how nutritional status and specific nutrients influence ISC dynamics (8, 11, 28). Food provides a key stimulus for preserving the growth and normal crypt-villus architecture of the GI epithelium (19). Hence, atrophy of the GI mucosa is a common gut mani-

festation of both undernutrition and exclusive parenteral nutrition in humans and laboratory animals (24).

Several gut trophic nutrients, including select amino acids, have been shown to promote GI epithelial homeostasis and barrier function in human and animal models of disease (3, 29). Among the amino acids with purported gut trophic effects, L-glutamine (Gln) continues to be a topic of keen interest, both as an important fuel for enterocytes and, more controversially, as a “conditionally essential” nutrient for GI epithelial homeostasis during severe illness (26). Despite several advances in understanding mechanisms of Gln in the gut (9), specific effects of Gln on ISC activation and differentiation have yet to be elucidated. This gap in knowledge is due, in part, to the long and elusive search for bona fide markers of ISCs. Thus the identification of a definitive marker for actively cycling ISCs (Lgr5) (2), along with the development of ex vivo intestinal organoid systems to study ISC dynamics (17), provides new approaches to address this question.

To elucidate the role of ISCs in Gln-mediated intestinal homeostasis, we examined jejunal crypt dynamics in undernourished wild-type mice treated with L-alanyl-L-glutamine (Ala-Gln), a stable Gln dipeptide shown to improve gut integrity in undernourished children and animal models (7, 23). Enteroids are a non-transformed tissue culture system containing ISCs and differentiated intestinal epithelial cells within an organotypic three-dimensional structure containing crypt-, villus-, and luminalike domains (12, 17). In addition, we tested whether Gln activates ERK and mammalian target of rapamycin (mTOR) signaling pathways in enteroids, as previously shown in transformed intestinal epithelial cell lines (6, 13, 14). Here, we report that 1) Ala-Gln protects mice against undernutrition-associated reductions in intestinal crypt numbers, 2) Ala-Gln and Gln promote proliferation of jejunal enteroid crypt domains, 3) Gln deprivation promotes a reversible quiescence of ISCs, and 4) Gln and Ala-Gln activate mTOR signaling in enteroids.

METHODS

Mouse model of undernutrition. We randomized wild-type C57/B6 dams with 10-day-old suckling pups to a multifactorial regional basic
diet (RBD) or an isocaloric, balanced control diet (CD), as previously described (22, 23). Pups were weaned to their dams’ diet (RBD vs. control) at 21 days of age and then sub randomized to ad libitum oral supplementation with a 111 mM solution of Ala-Gln (AminoStable, Ajinomoto) or plain drinking water. We maintained weanlings on the specified diets and treatments for 3 wk, until euthanization at 6 wk of age. We then harvested jejunal tissue for histological comparisons of numbers of crypts per millimeter of intestine. A microscopist (K.J.B.) counted ten 1-mm linear sections at ×20 magnification per mouse in a masked manner. Mean counts per millimeter were compared between well-nourished controls (n = 3), undernourished mice treated with Ala-Gln (n = 6), and undernourished untreated mice (n = 3). All animal protocols were approved by the Cincinnati Children’s Hospital Medical Center Animal Care and Use Committee.

Enteroids. Using a method described previously (10, 17), we prepared enteroids by isolating fresh midjejunal crypts from wild-type or EGFP-Lgr5 mice. Animals were killed by CO2 inhalation followed by cervical dislocation. We dissected ~5 cm of jejunum, flushed the tissue with ice-cold PBS, and splayed the tissue open. The sliced segments were cut into 1-cm pieces and transferred to 5 ml of cold PBS on ice. This suspension was placed on a rocking table at 4°C for 5 min for removal of residual blood or stool from jejunal segments. After the suspension was rocked, the PBS was aspirated, and 5 ml of 2 mM EDTA chelation buffer were added. The suspension was returned to a rocking table at 4°C for 30 min. Chelation buffer was removed, and 5 ml of shaking buffer (PBS with 43.3 mM sucrose and 54.9 mM sorbitol) were added. The suspension, in a conical tube, was gently shaken by hand for 2 min. A sample of the crypt suspension was visualized using a microscope to ensure that crypts had been released. If crypts were still attached, samples were gently shaken for an additional 0.5–2 min. The intestinal crypt suspension was filtered through a 70-μm cell strainer into a 50-ml conical tube. The filter was rinsed with 5 ml of cold shaking buffer. Samples were visualized again, and a portion of the suspension was centrifuged for 10 min at 4°C. The supernatant was gently poured off, with care taken to ensure that all excess liquid was removed from the tube. Intestinal crypts were resuspended in Matrigel (BD Biosciences, San Jose, CA) plus the growth factors R-spondin, mouse Noggin, and mouse epidermal growth factor (EGF; R & D Systems, Minneapolis, MN) and then plated into tissue culture plates. The Matrigel suspension was allowed to polymerize at 37°C for 0.25–1 h before fresh minigut medium (Advanced DMEM/F12 supplemented with 100 μM penicillin-100 μg/ml streptomycin, 10 mM HEPES, N-2, and B-27, in the absence or presence of 2 mM Gln) was supplied. Minigut medium and growth factors were replaced every 3–4 days. For passage of enteroids, the plates were rinsed with ice-cold PBS and cold PBS was added; a P1000 pipette tip was used to scrape the Matrigel from the vessel surface and break apart enteroids. Intestinal enteroid-Matrigel suspensions were pooled and spun down at 4°C for 10 min at 150 g. Enteroids were resuspended in 1–2 ml of cold PBS and passed through a syringe needle once to further break apart crypts. Enteroids were centrifuged again at 150 g to pellet the suspension. Matrigel with growth factors was used to resuspend the crypts, which were then reapplied to tissue culture plates. The medium used for the enteroids contained 2 mM Gln (standard media conditions) or 0 mM Gln (Gln-free medium). For single Lgr5-positive ISC experiments, ISCs were isolated following the methods of Sato and colleagues (17). Single crypts were released from the small intestine of EGFP-Lgr5 mice by incubation for 30 min at 4°C in PBS containing 2 mM EDTA. Isolated crypts were incubated in culture medium for 45 min at 37°C. After dissociation, the cells were passed through a cell strainer with a pore size of 20 μm. Green fluorescent protein (GFP)-positive cells were sorted by flow cytometry. Briefly, EGFP-Lgr5-positive cells were sorted and cultured as previously described (10). The FACS Aria II was equipped with a 100-μm nozzle (BD Biosciences). Enhanced GFP (EGFP)-positive cells were sorted, collected, pelleted, and embedded in Matrigel supplemented with 500 ng/ml EGF, 100 ng/ml Noggin, and 500 ng/ml Jagged-1 Fc chimera (R & D Systems). Each well was overlaid with culture medium containing 1 mM N-acetylcysteine (Sigma), 2.5 μM thiazovivin, and 2.5 μM CHIR99021 (Stemgent, Cambridge, MA). At 2 days after the cells were sorted, culture medium was replaced with culture medium supplemented with 1 μg/ml R-Spondin 1 (Preprotech), 500 ng/ml Jagged-1 Fc chimera, 50 ng/ml EGF, and 100 ng/ml Noggin (R & D Systems). Sorted cells were embedded in Matrigel containing organoid growth factors, as well as Y-27632 (10 μM) to avoid anoikis. ISCs were allocated to standard medium containing 2 mM Gln or Gln-free medium for 24, 48, 72, or 168 h and observed serially by light microscopy for expansion and crypt budding.

Microscopy. To capture differences in epithelial proliferation, enteroids were plated with standard or Gln-deprived medium for 24 h. A third group of enteroids was imaged at 3 h following Gln rescue after 24 h of Gln deprivation. Enteroids were fixed in 2% paraformaldehyde and stained with 5-ethyl-2'-deoxyuridine (EdU) according to the protocol suggested by the manufacturer (Click-it EdU Cell Proliferation Assay, Invitrogen). Similarly, fluorescent antibodies to mucin-2 (Life Technologies) and lysozyme (Life Technologies) were used to stain for goblet and Paneth cells, respectively. Images were captured by confocal microscopy (Zeiss LSM 710).

Western blot analysis. Enteroids were plated onto 12-well dishes with Gln-deprived medium for 24 h. At 24 h, PBS, Ala, Gln, or Ala-Gln (2 mM, the standard Gln concentration in minigut medium) was added; after 1 h, enteroids were lysed in RIPA buffer. We selected this time point to identify signaling events that precede the proliferative effects of Gln shown in Fig. 4 on the basis of previous work by Larson et al. (6) in transformed rat intestinal epithelial cells. Protein samples were prepared in 1× SDS sample buffer, run into Bio-Rad mini-PROTEAN 4–15% TGX gels, and then transferred onto nitrocellulose membranes using the Bio-Rad Trans-Blot Turbo system. The following antibodies were used: ERK1/2 (1:1,000 dilution; Cell Signaling Technology), phosphorylated ERK1/2 (1:1,000 dilution; Cell Signaling Technology), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1, 1:1,000 dilution; Cell Signaling Technology), phosphorylated 4E-BP1 (1:1,000 dilution; Cell Signaling Technology), ribosomal protein S6 kinase (p70S6K, 1:1,000 dilution; Cell Signaling Technology), phosphorylated p70S6K (1:1,000 dilution; Cell Signaling Technology), S6K1 (1:1,000 dilution; Cell Signaling Technology), phosphorylated S6K1 (1:1,000 dilution; Cell Signaling Technology), and actin (1:500 dilution; Developmental Studies Hybridoma Bank). Antibody detection was performed using the LI-COR Odyssey system. Each well was filled with the total amount of protein for each group, and expression levels were normalized to actin expression. Experiments were performed on three wells per group in triplicate.

Statistical analysis. We performed statistical tests using GraphPad Prism (version 5.0b, GraphPad, La Jolla, CA). Values are means ± SE. Statistical significance was determined by one-way or repeated-measures ANOVA, with corrections for multiple comparisons as appropriate. P < 0.05 was considered statistically significant. All in vitro and in vivo experiments were performed at least three times.

RESULTS

Ala-Gln protects against jejunal crypt depletion in undernourished mice. We allocated dams of 10-day-old suckling pups to a multideficient RBD (kcal%: 88% carbohydrates, 7% protein, and 5% fat) or an isocaloric CD (kcal%: 65% carbohydrate, 20% protein, and 15% fat) (22). Pups were weaned at 21 days of age to their dams’ diet and randomized to plain drinking water or 111 mM Ala-Gln solution. We previously reported the composition of the RBD and protective effects of Ala-Gln on jejunal villus height, crypt depth, barrier function, and catch-up growth in RBD-challenged mice (23). In the
current study we detected no differences in weight-based food consumption between groups (2.48 ± 0.11, 2.61 ± 0.37, and 2.64 ± 0.48 g feed·g body wt⁻¹·day⁻¹ in CD, RBD, and RBD + Ala-Gln, respectively; \( P = 0.66 \), by ANOVA). However, we observed significant differences in weight-based fluid consumption (5.7 ± 0.2, 9.4 ± 1.1, and 11.6 ± 2.2 ml·g body wt⁻¹·day⁻¹ in CD, RBD, and RBD + Ala-Gln, respectively; \( P < 0.05 \) for CD vs. either RBD or RBD + Ala-Gln, by ANOVA with Bonferroni’s correction for multiple comparisons).

Histological examination of jejunal segments from RBD mice euthanized at 6 wk of age revealed a 20% decrease in the number of crypts per length of mucosa in undernourished mice compared with well-nourished controls, a decrease not seen in RBD mice treated with Ala-Gln (Fig. 1; \( P < 0.001 \), by ANOVA with Bonferroni’s correction for multiple comparisons). These results demonstrate that Gln is sufficient to restore the normal density of crypts in this developmental model of nutritional deprivation and suggest that Gln functions to enhance stem cell expansion in the intestine.

Gln and Ala-Gln enhance crypt expansion in mouse jejunal organoids. For determination of the effects of Gln on crypt dynamics ex vivo, we used standard minigut medium containing 2 mM Gln to generate enteroids from the jejunal crypts of undernourished mice compared with well-nourished controls, a decrease not seen in RBD mice treated with Ala-Gln (Fig. 1; \( P < 0.001 \), by ANOVA with Bonferroni’s correction for multiple comparisons). Consistent with our in vivo findings (see above), we recovered fewer jejunal crypts from undernourished mice than nourished controls (data not shown). Crypts isolated from undernourished and nourished mice were equally viable and expanded to develop enteroids with multiple crypt domains and a stable crypt-villus structure. We conducted all subsequent experiments using jejunal tissue from well-nourished mice.

To define the effects of select nonessential amino acids on rates of crypt budding, we passaged enteroids derived from a single mouse into Gln-free medium or medium supplemented with Gln, Ala, Ala-Gln, asparagine, glutamate, glycine, or serine at 2 mM, the standard Gln concentration in minigut medium. From these, we identified enteroids with a maximum of one or two crypts and then measured formation of new crypts at 24-h intervals over the next 4 days to calculate a crypt expansion index (number of crypts at day x / number of crypts at day 0). As shown in Fig. 2, expansion of crypt domains was two- to threefold greater in the first 48 h in enteroids maintained in Ala-Gln or Gln than in enteroids maintained in Gln-free medium or Gln-free medium supplemented with other nonessential amino acids (\( P < 0.001 \) for Ala-Gln or Gln vs. other media, by repeated-measures ANOVA with Bonferroni’s correction for multiple comparisons).

Gln deprivation halts enteroid proliferation and induces reversible crypt atrophy. To observe the effects of Gln deprivation on the morphology of fully expanded enteroids containing multiple crypt domains, we substituted standard 2 mM Gln enteroid medium with Gln-free medium. Qualitatively, enteroids maintained in the absence of Gln showed a progressive atrophy of crypt domains over 48 h (Fig. 3, A–C). Reintroduction of Gln after 48 h reversed these effects, as evidenced by a gradual expansion of previously atrophied crypt domains and formation of new crypt domains within 48 h (Fig. 3, D and E). Crypt atrophy in the absence of Gln was accompanied by a decrease in epithelial proliferation (Fig. 4) as measured by incorporation of EdU following 24 h of Gln deprivation. Furthermore, Gln rescued normal levels of proliferation within 3 h (Fig. 4, C and D). Despite crypt atrophy and decreased
proliferation, the relative abundance of Paneth and goblet cells remaining stable following 24 h of Gln deprivation (Fig. 5).

We next examined the effects of Gln deprivation on expression of the ISC marker Lgr5 using enteroids derived from EGFP-Lgr5 mice (17). Depriving enteroids of Gln beyond 72 h led to enteroid collapse, with extrusion of lumen contents into the surrounding medium (Fig. 6, A–D). We did not detect significant fluctuations in EGFP-Lgr5 expression in response to the presence or absence of Gln. Despite enteroid collapse, viable ISCs persisted up to 72 h following Gln deprivation, as determined by reorganization of enteroids within 48 h of Gln rescue (Fig. 6, E and F). Intriguingly, single Lgr5-positive ISCs harvested from EGFP-Lgr5 mice and isolated by flow cytometry did not develop crypt buds in the absence of Gln (Fig. 7, bottom), whereas single ISCs grown in the presence of Gln expanded to form organoids (Fig. 7, top). Together, these results demonstrate that Gln is an essential nutrient that supports the ability of Lgr5-positive ISCs to increase proliferation and crypt expansion in enteroids.

Gln or Ala-Gln rescue activates mTOR pathways. To assess the possible involvement of mTOR and ERK1/2 signaling pathways in the epithelial proliferative response to Gln in enteroids, we deprived enteroids of Gln for 24 h then treated them with Ala, Gln, Ala-Gln (all at a final concentration of 2 mM), or a PBS vehicle control for 1 h, a time point previously shown to reveal Gln-stimulated proliferative signaling in transformed rat intestinal epithelial cells (6). In enteroids treated with Gln and Ala-Gln (but not Ala or PBS), we observed a significant increase in phosphorylated p70 ribosomal protein S6 kinase (P-p70S6K) and phosphorylated ribosomal S6 protein (P-S6), as determined by Western blotting. These results indicate that Gln stimulates activation of mTOR signaling in enteroids. In contrast, Ala-Gln alone modestly activated 4E-BP1 and ERK1/2 signaling compared with PBS controls (Fig. 8).

DISCUSSION

In this study, using ex vivo mouse jejunal enteroids to complement an in vivo finding of crypt preservation in undernourished mice treated with oral Ala-Gln, a stable dipeptide previously shown to promote growth and gut integrity in undernourished children and mice (7, 23), we demonstrate important effects of Gln and Ala-Gln on intestinal epithelial homeostasis. In enteroids, we found that Gln or Ala-Gln promoted mTOR signal activation, epithelial proliferation, and crypt expansion. Conversely, withdrawal of Gln from fully expanded enteroids reduced enterocyte proliferation, induced crypt atrophy, and led to the eventual collapse of enteroids. Importantly, these effects were reversible with Gln rescue, with ISCs demonstrating the capacity to reactivate following prolonged Gln deprivation.

Gln, the most abundant amino acid in serum, is an important substrate and signaling molecule for intestinal epithelial cell proliferation and survival (9). A Gln-enriched diet has previously been shown to ameliorate malnutrition-related enteropathy in rats (21). Additional work utilizing transformed intestinal epithelial cell lines from rats, pigs, or humans identified several cytoprotective or cell death pathways modulated by Gln, including ERK1/2 (6, 14, 15), phosphatidylinositol-3/Akt (6), mTOR (13), EGF receptor (18), heat shock proteins (5, 27), and Sp3 (1), as well as signals that regulate autophagy (16). Using PBS as a vehicle control and Ala as an amino acid control, we found that Gln and Ala-Gln, but not Ala, activated the mTOR signaling proteins p70S6K and S6 at 1 h in non-transformed, Gln-starved enteroids. Activation of mTOR signaling pathways in enteroids in response to a mixture of amino acids that included Gln has previously been described (28). Our findings indicate that, in ISCs, Gln may be a key activator of mTOR, a major pathway involved in the regulation of cell cycle and protein synthesis by amino acids. mTOR plays a critical role in cell cycle progression by phosphorylating and activating p70S6K and 4E-BP1, two major proteins involved in the regulation of protein synthesis (4).
Importantly, we determined that Lgr5-positive ISCs appeared resilient to the adverse effects of Gln deprivation, remaining viable but quiescent in culture and reactivating when Gln was reintroduced to the medium. We could not recapitulate Gln or Ala-Gln effects by isomolar substitutions of other nonessential amino acids or with isoenergetic amounts of glucose (data not shown). We previously showed that Ala-Gln supplementation protects mice against crypt and villus atrophy via modulation of epithelial proliferation, an effect we have also demonstrated in conditionally immortalized mouse small intestinal epithelial cells (23). The current study builds on and extends this earlier report by demonstrating similar effects of Gln and Ala-Gln in enteroids, with the novel finding that Gln deprivation induces quiescence of individual ISCs and atrophy of cryptlike domains in fully expanded enteroids.

Enteroids offer several potential advantages over cultured epithelial monolayers for further study of Gln effects and mechanisms in the gut. 1) Enteroids are derived from primary, nontransformed intestinal epithelial stem cells and, as such, may have greater in vitro relevance to in vivo processes. 2) The crypt-villus structure of enteroids allows real-time assessment of the morphological effects of amino acid deprivation and supplementation. 3) Enteroid ISC's differentiate into all epithelial lineages and, thus, will facilitate future studies of Gln effects on ISC differentiation, although we saw no short-term effects on differentiation in the current study. 4) Enteroids display robust circadian rhythms that allow more appropriate modeling of the effects of nutrition on ISC dynamics (12). 5) Recently published reports describing the generation of intestinal organoids from human pluripotent stem cells and human intestinal biopsies will accelerate future efforts to confirm the present findings in human intestinal organoids (20, 25).

There are several caveats to consider in interpreting our findings. 1) Because RBD mice do not survive weaning when housed individually, we did not perform pair-feeding experiments or obtain precise measures of postweaning fluid and food intake. Thus, although we utilized isocaloric diets, uncontrolled group differences in food or fluid consumption might have confounded our in vivo finding of jejunal crypt preservation in RBD mice treated with Ala-Gln. On the basis of body weight-adjusted estimates of fluid intake, we detected a statistically significant difference in fluid consumption between CD mice and either RBD or RBD/H11001 Ala-Gln mice but no difference between RBD and RBD/H11001 Ala-Gln mice. Furthermore, we detected no group differences in body weight-adjusted food consumption. However, because of the lack of an amino acid control solution for Ala-Gln in our in vivo experiments, it is impossible to dissect the extent to which Ala-Gln modified jejunal crypt dynamics via its role as an energy source or as an intestinal epithelial cell mitogen. On the basis of the fluid intake of RBD + Ala-Gln mice, we estimated a daily Ala-Gln consumption of 1.29 mmol/g body wt (0.28 g Ala-Gln/g body wt)

Fig. 4. Gln deprivation decreases proliferation of intestinal epithelial cells in mouse jejunal enteroids. A and B: representative confocal 3-dimensional reconstruction images of 5-ethyl-2'-deoxyuridine (EdU) incorporation in enteroids maintained for 24 h in the presence of 2 mM Gln (Gln+) or the absence of Gln (Gln−). C: a third group of enteroids imaged 3 h following Gln rescue after 24 h of Gln deprivation. D: proportion of EdU-positive cells. Values are means ± SE. *P < 0.01 (by ANOVA with Bonferroni's correction).
For Ala-Gln-treated RBD mice, we estimated that Ala-Gln consumption provided an additional 1.12 kcal·g body wt−1·day−1, or an additional 11.3% increase in daily calories. Hence, future in vivo studies to rigorously distinguish the effects of Ala-Gln’s caloric contribution vs. growth factor-like activity would require isocaloric control solutions, e.g., Ala or di-Ala, to address this important question. Isomolar substitution of nonessential amino acids for Gln did not rescue the adverse effects of Gln deprivation in enteroid experiments (Figs. 2 and 8), indicating that Gln’s trophic effect on intestinal cells may be distinct from Gln’s role as an energy source (9).

We found that Gln and Ala-Gln activate mTOR signaling in enteroids, but we did not establish a requirement for these pathways in mediating the proliferative effects of Gln and Ala-Gln. In enteroid models of amino acid deprivation, Efeyan and colleagues (4) convincingly demonstrated that mTORC1 is a key mediator of the coupling between amino acid metabolism and ISC activation. 2) We acknowledge that our observations in enteroids (Figs. 3, 6, and 7) are limited by their qualitative nature. Quantifying individual crypt formation and dimensions in both mature 3D enteroids (Figs. 3 and 6), which contain an abundance of overlying cryptlike domains, and single EGFP-Lgr5-positive stem spheres (Fig. 7), which may not always form cryptlike domains, presented technical diffi-
cultivates. Given these hurdles, we elected to measure crypt formation in newly generated enteroids containing one or two cryptlike domains, thereby facilitating the identification of new crypts every 24 h (Fig. 2). 4) The abundance and batch-to-batch consistency of the free Gln or Gln oligopeptide content in Matrigel is unknown. 5) Our in vivo findings were the result of orally administered Ala-Gln, which might exert luminal and systemic effects on ISCs. In contrast, our ex vivo results represent the addition Gln or Ala-Gln to enteroid medium alone, and not to the enteroid lumen. Nonetheless, we did observe complementary effects in both models. Furthermore, reversal of gut mucosal atrophy has been reported with addition of Gln to exclusive parenteral nutrition in patients (24).

Although the specific mechanisms remain unclear, our current in vitro findings suggest that mTOR signaling is associated with Lgr5-positive ISC proliferation and crypt expansion. In vivo, this mechanism would provide a clear survival advantage by allowing mammals to couple ISC cycling and crypt-villus surface area to the availability of food. In this model, ISCs become dormant when food is scarce, leading to decreased epithelial proliferation and mucosal atrophy. Conversely, when food is abundant, ISCs reanimate to replenish the epithelium, thus allowing optimal nutrient absorption. It is possible that this adaptive atrophy is more pronounced in mice, which are episodic feeders in the wild, than in humans, who eat ad libitum. A more complete understanding of these mechanisms...
should lead to novel interventions that enhance recovery from intestinal injuries in the setting of undernutrition [e.g., as has been shown with Ala-Gln in improving gut integrity and catch-up growth in Brazilian children with malnutrition enteropathy (7)] and identification of strategies to induce quiescence of aberrantly activated ISCs to prevent or treat GI cancers or protect healthy ISCs in the setting of chemoradiotherapy (4).

We conclude that Glu and Ala-Gln activate ISCs via the mTOR pathway to promote epithelial proliferation and preserve intestinal crypts. Future studies are needed to address the caloric vs. mitogenic properties of Glu and Ala-Gln in ameliorating effects of undernutrition on the gut. Further studies are also needed to determine the specificity of these findings to Lgr5-positive ISCs vs. other putative ISC populations in the gut, exploit enteroids as a platform for the study of intestinal epithelial differentiation under nutritional stress, verify findings in human intestinal organoids and enteroids, and ultimately define the mechanisms by which Glu regulates ISC cycling to promote GI homeostasis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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