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Effect of essential amino acids on enteroids: Methionine deprivation suppresses proliferation and affects differentiation in enteroid stem cells

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ABSTRACT

We investigated the effects of essential amino acids on intestinal stem cell proliferation and differentiation using murine small intestinal organoids (enteroids) from the jejunum. By selectively removing individual essential amino acids from culture medium, we found that 24 h of methionine (Met) deprivation markedly suppressed cell proliferation in enteroids. This effect was rescued when enteroids cultured in Met deprivation media for 12 h were transferred to complete medium, suggesting that Met plays an important role in enteroid cell proliferation. In addition, mRNA levels of the stem cell marker *leucine-rich repeat-containing G protein-coupled receptor 5* (*Lgr5*) decreased in enteroids grown in Met deprivation conditions. Consistent with this observation, Met deprivation also attenuated Lgr5-EGFP fluorescence intensity in enteroids. In contrast, Met deprivation enhanced mRNA levels of the enteroendocrine cell marker *chromogranin A* (*ChgA*) and markers of K cells, enterochromaffin cells, goblet cells, and Paneth cells. Immunofluorescence experiments demonstrated that Met deprivation led to an increase in the number of ChgA-positive cells. These results suggest that Met deprivation suppresses stem cell proliferation, thereby promoting differentiation. In conclusion, Met is an important nutrient in the maintenance of intestinal stem cells and Met deprivation potentially affects cell differentiation.

1. Introduction

The small intestine is an organ with various functions, including nutrient absorption, protection against pathogens and xenobiotics, signal recognition, and the production of bioactive compounds [1]. These functions are mediated by mature intestinal epithelial cells, including enterocytes, enteroendocrine (EE) cells, goblet cells, and Paneth cells, all of which arise from Lgr5-positive stem cells. Enterocytes, goblet cells, and EE cells localize to villi protruding toward the intestinal lumen, whereas Paneth cells and stem cells localize to the crypt, which is the pitted area of the intestinal lumen [2]. Stem cells in the small intestine not only continuously self-replicate throughout their lifetime, they also differentiate into multiple types of epithelial cells [2]. Enterocytes are the primary cell type in the intestinal epithelium, and they play a prominent role in the digestion and absorption of nutrients [2]. Goblet cells secrete polysaccharides to form a mucin layer that protects against the invasion of pathogens by blocking their attachment to the epithelium [2]. Although EE cells are a less abundant cell type, they detect saccharides and fatty acids from the diet and secrete various hormones that regulate the production of essential digestive enzymes and promote bowel movements [2]. Paneth cells localize to the bottom of the crypt, where they are protected from bacterial invasion by antibacterial peptides such as α-defensin [2,3]. In addition, along with subepithelial fibroblasts, Paneth cells constitute the stem cell niche and play an important role in maintaining stem cell function by transmitting Notch signals to stem cells [2,4].

Dietary amino acids play a key role in maintaining gut health and preventing intestinal diseases [5]. For example, glutamine protects against jejunal crypt depletion in undernourished mice [6].

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List of abbreviation

ChgA	Chromogranin A
CM	Conditioned medium
DOT1L	Disruptor of telomeric silencing 1-like
DW	Distilled water
EdU	5-ethynyl-2-deoxyuridine
EE	Enteroendocrine
Gip	Glucose-dependent insulinotropic polypeptide
HBSS	Hanks' balanced salt solution
I-FABP	Intestinal fatty-acid-binding protein
ITF/TFF3	Intestinal trefoil factor/Trefoil factor 3
Lgr5	Leucine-rich repeat-containing G protein-coupled receptor 5
Lyz1	Lysozyme 1
Met	Methionine
PBS	Phosphate-buffered saline
RT-PCR	Reverse transcription-polymerase chain reaction
SAM	S-adenosilmethionine
SE	Standard error
Tph	Tryptophan 5-hydroxylase

However, investigating the role of individual amino acids in intestinal cell function and proliferation *in vivo* is challenging. Furthermore, maintaining primary cultures of fully functional intestinal epithelial cells is difficult due to their relatively slow growth rate and the lack of information regarding the process by which small intestinal cells differentiate [1]. Therefore, intestinal function has primarily been studied in transformed cell lines, including Caco-2 cells. However, these cell lines lack the cellular complexity and transport activity associated with native small intestinal tissue [7]. In 2009, Sato et al. [8] established the three-dimensional intestinal organoid culture system, which mimics villi-crypt structures. These intestinal organoids (enteroids) comprise stem cells and mature intestinal epithelial cells, both of which replicate the *in vivo* functions of these cell types. Thus, studies using enteroids are expected to provide new insights into the intestine. However, the effect of specific nutrients on intestinal organoids remains unclear [9,10].

In this study, we focused on the effect of amino acids on enteroids generated from the mouse jejunum. Amino acids might affect the self-renewal and differentiation potential of intestinal stem cells, as protein starvation reduces the number of intestinal stem cells [11]. There has only been one report demonstrating that the non-essential amino acids Gln and Ala-Gln promote epithelial proliferation and crypt expansion in enteroids [6]. Essential amino acids cannot be synthesized by organisms and must be obtained by ingestion. We hypothesized that essential amino acids have a greater impact on enteroids compared with non-essential amino acids. Therefore, the present study focused on the effects of essential amino acids. As basic enteroid culture medium is abundant in essential amino acids, we evaluated 10 different media, each of which lacked a single essential amino acid.

We found that methionine deprivation led to a decrease in the number of stem cells and potentially enhanced cell differentiation into EE cells, goblet cells, and Paneth cells in murine enteroids.

2. Material and methods

2.1. Animals

C57BL/6J (wild-type) mice (CLEA Japan, Inc., Tokyo, Japan) and Lgr5-EGFP-IRES-creERT2 (Lgr5-EGFP) mice (The Jackson Laboratory, Bar Harbor, ME) were used in the study. Mice were housed in standard plastic cages in an animal facility maintained at 23–25 °C and 50–56% humidity under a 12 h light/12 h dark cycle (lights on 8:00–20:00), and they had free access to tap water and standard laboratory rodent food (Oriental Yeast Co., Ltd., Tokyo, Japan). The animal experiments adhered to the guidelines for the maintenance and handling of experimental animals established by the Tokyo University of Agriculture Ethics Committee.

2.2. Enteroid culture

Enteroids were isolated from fresh jejunal crypts obtained from wild-type or Lgr5-EGFP mice as previously described by Sato et al. [8] with some modifications. The animals were euthanized by cervical dislocation. After the animals were dissected, 5–6 cm of the jejunum was collected and the luminal contents were thoroughly washed with ice-cold phosphate-buffered saline (PBS). The jejunum was longitudinally opened and placed on a cold stainless steel tray with the luminal side facing upward. After the villi were scraped with a scalpel, the jejunum was agitated in 10 mL of 30 mM EDTA-3Na/Hanks' balanced salt solution (HBSS) for 10 min at room temperature. The jejunum was placed into a tube containing 10 mL of 30 mM EDTA-3Na/HBSS. The tube was shaken 20 times, and the first fraction was subsequently collected. Then, the jejunum was transferred to a separate tube containing 10 mL of ice-cold HBSS. The tube was shaken 50 times, and the second fraction was subsequently collected. Likewise, the third and the forth fractions were collected by transferring the jejunum to a tube containing 10 mL of ice-cold HBSS, and the tube was shaken 50 and 100 times, respectively. Each fraction was centrifuged at 400 × g at 4 °C for 4 min, and the supernatant was subsequently removed. Pellets from each fraction were individually suspended in 500 μL of 10 μM Y-27632/HBSS. After counting crypts and villi using a microscope, crypt purity was calculated using the following formula: crypt purity (%) = crypts/(villi + crypts). Samples with >80% purity were centrifuged at 400 × g at 4 °C for 4 min. The supernatants were removed, and the crypts (300 crypts/well) were embedded in 60% Matrigel® (BD Biosciences, San Jose, CA) (60 μL/well) in a 12-well plate (Nippon Gene Co., Ltd., Tokyo, Japan). The Matrigel® suspension was allowed to polymerize at 37 °C for 10 min before fresh Advanced DMEM/F12 supplemented with Glutamax™, N-2, B-27 (Thermo Fisher Scientific Inc., Waltham, MA), penicillin/streptomycin, 10 mM HEPES (NACALAI TESQUE, Inc., Kyoto, Japan), 10% R-spondin conditioned medium (CM), 5% Noggin CM, and 50 ng/mL recombinant murine EGF (Funakoshi Co., Ltd., Tokyo, Japan) was added. This medium was defined as basic medium. The basic medium was replaced every 2–3 days. Enteroids from wild-type mice were collected using ice-cold PBS. They were mechanically disrupted by passage through a syringe with a needle (27G) (Terumo Corporation, Tokyo, Japan) and subsequently transferred to fresh 60% Matrigel®. The enteroids were passaged every 4–6 days. Enteroids from Lgr5-EGFP mice were freshly prepared for each use because their fluorescence intensity diminished with each passage.

2.3. 5-ethynyl-2-deoxyuridine (EdU) assay

Enteroids cultured with basic medium for 3 days were placed into the wells of a Nunc™ Lab-Tek™ II 8-well glass bottom chamber (Thermo Fisher Scientific Inc.) (approximately 20 enteroids/well) and resuspended in 60% Matrigel® (5 μL/well). After 10 min of Matrigel® polymerization, 200 μL of Advanced DMEM/F12 (Research Institute for the Functional Peptides Co., Ltd., Yamagata, Japan) or 200 μL of Advanced DMEM/F12 lacking individual essential amino acids (-valine, -leucine, -isoleucine, -threonine,