Ex Vivo Enteroids Recapitulate In Vivo Citrulline Production in Mice

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Abstract

Background: The endogenous production of arginine relies on the synthesis of citrulline by enteral ornithine transcarbamylase (OTC). Mutations in the gene coding for this enzyme are the most frequent cause of urea cycle disorders. There is a lack of correlation between in vivo metabolic function and DNA sequence, transcript abundance, or in vitro enzyme activity.

Objective: The goal of the present work was to test the hypothesis that enteroids, a novel ex vivo model, are able to recapitulate the in vivo citrulline production of wild-type (WT) and mutant mice.

Methods: Six-week-old male WT and OTC-deficient mice [sparse fur and abnormal skin (*spf-ash*) mutation] were studied. Urea and citrulline fluxes were determined in vivo, and OTC abundance was measured in liver and gut tissue. Intestinal crypts were isolated and cultured to develop enteroids. Ex vivo citrulline production and OTC abundance were determined in these enteroids.

Results: Liver OTC abundance was lower (mean ± SE: 0.16 ± 0.01 compared with 1.85 ± 0.18 arbitrary units; $P < 0.001$) in *spf-ash* mice than in WT mice, but there was no difference in urea production. In gut tissue, OTC was barely detectable in mutant mice; despite this, a lower but substantial citrulline production (67 ± 3 compared with 167 ± 8 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1}; $P < 0.001$) was shown in the mutant mice. Enteroids recapitulated the in vivo findings of a very low OTC content accompanied by a reduced citrulline production (1.07 ± 0.20 compared with 4.64 ± 0.44 nmol $\cdot$ μg DNA^{-1} $\cdot$ d^{-1}; $P < 0.001$).

Conclusion: Enteroids recapitulate in vivo citrulline production and offer the opportunity to study the regulation of citrulline production in a highly manipulable system. *J Nutr* 2018;148:1415–1420.

Introduction

Although the urea cycle and the pathway for the endogenous synthesis of arginine share many enzymes and transporters, they have different functional goals. Although the urea cycle functions to detoxify ammonia with all its components occupying a well-defined compartment spanning the mitochondrial membrane (1), the pathway for arginine synthesis is an interorgan process geared toward the production of this semi-indispensable amino acid (2). Both pathways start with the production of citrulline by carbamylation of ornithine by ornithine transcarbamylase (OTC), which is only expressed in hepatocytes and enterocytes (3, 4). In hepatocytes, the citrulline produced stays within the cycle to yield urea and regenerate ornithine. In contrast, citrulline synthesized in enterocytes is exported into portal blood and is available to tissues and organs (mainly the kidney) for arginine synthesis (2).

Mutations of the OTC gene are the most common urea disorder, with hundreds of distinct mutations reported (5) that result in a wide range of clinical presentations (6). Furthermore, because the OTC gene is located in the X chromosome, females are usually carriers and manifest the disorder later in life (7). The devastating consequences of impaired ammonia detoxification and hyperammonemia have made the restoration of the urea cycle the primary therapeutic target for urea cycle disorders (8). However, the increased survival of children with urea cycle disorders has created a new need for transitional care from infancy to adulthood (9). Among those needs, the maintenance of arginine supply is crucial to meet the multiple fates of this amino acid (10). Although the determination of citrulline production by the gut and de novo arginine synthesis is a

straightforward procedure (11), the manipulation of enteral citrulline production by metabolic or genetic means becomes rather complex in a whole organism.

In vitro studies are an attractive alternative to test different interventions, because many manipulations can be easily performed. However, transformed intestinal epithelial cell lines may not represent normal in vivo metabolism and primary cell cultures have a short life that prevents some interventions. In addition, these cell cultures rarely represent the whole population of epithelial cells that constitute the intestinal lining. In contrast, enteroids, a novel ex vivo model, are a nontransformed tissue culture system containing intestinal stem cells and differentiated intestinal epithelial cells within a 3-dimensional structure containing crypt-, villus-, and lumen-like domains that recapitulate the in vivo architecture of the small intestinal epithelium (12). However, little is known about the metabolism of enteroids (12, 13) and even less about citrulline production. In addition, because citrulline has been proposed as a marker of gut mass and function in vivo (14), it may serve the same role in this novel ex vivo system. Therefore, the goal of the present work was to test the hypothesis that ex vivo enteroids recapitulate the in vivo citrulline production of wild-type (WT) and sparse fur and abnormal skin (*spf-ash*) mice, a hypomorphic model of OTC deficiency.

Methods

Animals and housing. ICR$^{spf-ash}$ mice were obtained by backcrossing B6EiC3Sn *a/A-Otc$^{spf-ash}$*/J mice into an Institute of Cancer Research (ICR) background (15) for >30 generations. Mutant mice (*spf-ash*) on the ICR background have higher viability, growth rate, and fertility than mice on the original background and are able to maintain ureagenesis despite a nitrogen-load challenge (15). Six- to eight-week-old male WT and *spf-ash* littermate mice were studied. Mice had free access to an autoclaved pelleted feed (LabDiet 5V5R chow: fat, 59 g/kg; fiber, 24 g/kg; carbohydrate, 570 g/kg; crude protein, 180 g/kg; arginine, 7.5 g/kg; ash, 50 g/kg), and autoclaved reverse osmosis water was available at all times. Mice were under a 12-h light cycle (0600–1800) in a temperature-controlled (22° ± 2°C) and humidity-controlled (55% ± 5%) environment. All animal procedures were authorized by the Baylor College of Medicine Institutional Animal Care and Use Committee.

In vivo urea and citrulline production. After removing the feed at 0700 and a 3-h feed-deprivation period, mice (WT: $n = 11$; weight: 32.7 ± 0.5 g; *spf-ash*: $n = 9$; 27.5 ± 1.6 g) were fitted with tail-vein catheters and primed-continuously infused for 4 h with (guanidino)[$^{15}N_2$]arginine (prime: 45 μmol/kg; continuous: 45 μmol · kg^{-1} · h^{-1}), 5-[^{13}C]3,3,4,4[$^2H_4$]citrulline (prime: 8 μmol/kg; continuous: 8 μmol · kg^{-1} · h^{-1}), 3,3[$^2H_2$]tyrosine (prime: 10 μmol/kg; continuous: 10 μmol · kg^{-1} · h^{-1}), (ring)[$^2H_5$]phenylalanine (prime: 16 μmol/kg; continuous: 16 μmol · kg^{-1} · h^{-1}), and [$^{13}C^{18}O$]urea (prime: 100 μmol/kg; continuous: 100 μmol · kg^{-1} · h^{-1}). A blood sample was obtained from the submandibular bundle at the end of the infusion. We have shown that isotopic plateau enrichment of the metabolites of interest is achieved within the 4 h infusion (16, 17). Gut (proximal jejunum) and liver tissue were collected, and a random subset of these samples (4 for gut and 5 for liver) was analyzed to determine OTC abundance and localization.

Ex vivo citrulline production. Intestinal crypts from the proximal jejunum were collected from WT ($n = 4$) and *spf-ash* ($n = 4$) mice after euthanasia as described by others (18). Crypts were cultured in Matrigel (Becton Dickinson) and complete medium with growth factors (CMGF+; Digestive Disease Center, Baylor College of Medicine, Houston, Texas) at 37°C and 5% CO_2 in 6-well plates (passage zero).

CMGF+ media is composed of DMEM/Ham's F-12 supplemented with alanyl-glutamine, N-acetylcysteine, Spondin, Noggin, epithelial growth factor, and B-27 and N-2 Supplement (ThermoFisher) (19). Enteroids were passaged twice after 6 d in culture to obtain the passage 2 experimental enteroids. These enteroids were cultured in 24-well plates for 120 h in triplicate per mouse; media was changed at 48 and 96 h, and the 96–120 h media were saved for citrulline concentration analysis. Enteroids were collected at this time for DNA and OTC abundance determinations. Additional enteroids were cultured in a 4-well chamber slide system (ThermoFisher) under similar conditions for immunohistochemistry (18).

Analytical analysis. For OTC abundance, 10 mg homogenized frozen tissue or enteroids from 1 well were lysed in 300 μL radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.5% Na-deoxycholate; 0.1% sodium dodecyl sulfate; 150 mM NaCl; 2 mM EDTA; and 50 mM NaF) containing protease inhibitor (P3100-005; GenDEPOT) and phosphatase inhibitor (04906837001; Roche). Samples were centrifuged at 13,000 × g for 15 min after incubation for 20 min on ice at 4°C. Bicinchoninic acid protein assay was used for quantitation of protein, and 20 μg protein/sample (except for liver, 10 μg) was loaded in 4–20% sodium dodecyl sulfate polyacrylamide gel for electrophoresis and transferred to polyvinylidene difluoride membrane (Bio-Rad). After 1 h of blocking in 5% nonfat milk at room temperature, membranes were incubated with antibodies (OTC: GTX105140, dilution 1:6000; GeneTex, Inc.; β-actin: AM4302, dilution 1:5000; ThermoFisher Scientific) overnight at 4°C. Due to the low abundance of OTC in mutant *spf-ash* mice, gel overloading (60 μg protein) and a second OTC antibody (AV41766; Sigma-Aldrich) were also used. Membranes were incubated with secondary antibodies (sc-2305 and sc-2031; Santa Cruz Biotechnology) for 1 h at room temperature. After incubation with West-Q Pico ECL solution (dilution 1:2000; GenDEPOT) for 3 min, signals were detected by Quantity One (Bio-Rad), and data were normalized with β-actin and analyzed by ImageJ software (NIH) (20).

For immunohistochemistry, formalin-fixed samples were paraffin-embedded and 5-μm-thick sections were dewaxed; antigen retrieval was performed with sodium citrate (pH 6.0) for 20 min at 95°C. Sections were incubated in blocking buffer (5% horse serum in PBS with 0.5% Tween-20) for 30 min. OTC primary antibody (GTX105140) was applied to sections overnight at 4°C (dilution: 1:100). After washing with 1× PBS with Tween-20 for 3 × 10 min, secondary antibodies were applied to sections at room temperature for 1 h (Alexa Fluor 488 donkey anti-goat, A-11055, dilution of 1:1000; and Alexa Fluor 555 donkey anti-rabbit, A-31572, dilution of 1:1000; ThermoFisher Scientific). Sections were mounted with Vectashield mounting medium with 4', 6-diamidino-2-phenylindole (H-1200; Vector Laboratories, Inc.). Signal was detected by using a deconvolution microscope (DVLive fluorescence microscope; GE Healthcare). Enteroids were immunostained as described by Debnath et al. (21).

The amount of DNA per well of enteroids was assessed with Hoechst 33258 dye (ThermoFisher) and measured by fluorescence spectrophotometry (excitation: 350 nm; emission: 473 nm). In addition, 5-ethynyl-2′-deoxyuridine (ThermoFisher) staining to determine DNA synthesis was used to visualize proliferating cells. Isotopic enrichments and concentrations of citrulline and other amino acids were determined by heated electrospray ionization–LC–tandem MS as previously described (22).

Calculations. Citrulline, urea, and other amino acid fluxes were determined by the isotopic dilution of the respective infused tracers as previously reported (23). Enteroid citrulline production was determined by the accumulation of citrulline in the medium in a 24-h period and expressed on a per-microgram DNA basis.

Data analysis. Data were analyzed by utilizing the Proc Mixed procedure of SAS (version 9.2; SAS Institute), with genotype as the fixed effect of the model. All of the data are reported as means ± SEMs and differences were considered significant at $P < 0.05$.
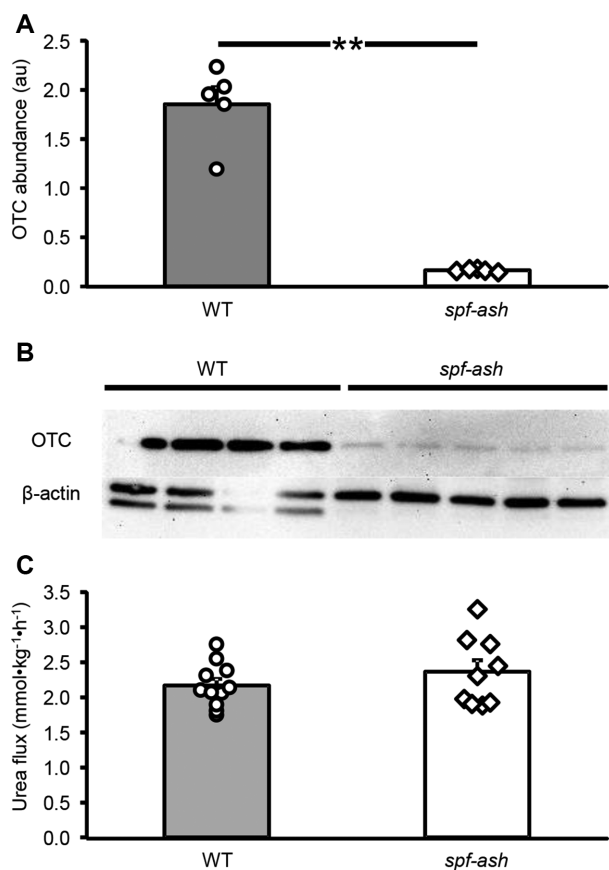
FIGURE 1 Liver OTC abundance (A), Western blot (B), and urea production (C) in ICR WT and ICR*spf-ash* mice. Values are means ± SEs; $n = 5$ for WT and *spf-ash* OTC abundance, $n = 11$ for WT, and $n = 9$ for *spf-ash* for urea flux. Individual values are also shown. **$P < 0.001$. au, arbitrary units; ICR, Institute of Cancer Research; OTC, ornithine transcarbamylase; *spf-ash*, sparse fur and abnormal skin; WT, wild-type.

Results

In vivo urea and citrulline production. A lower OTC abundance in the liver of *spf-ash* mice ($P < 0.001$; **Figure 1**A) measured by Western blot (Figure 1B) was observed, with only ~9% of residual abundance compared with WT control mice. Despite this finding, no differences ($P = 0.32$) in urea production were determined between the 2 genotypes. OTC abundance in intestinal tissue was barely detectable in *spf-ash* mice (**Figure 2**A, B). Increasing the protein load in the Western blot analysis (**Supplemental Figure 1**) or the use of a second OTC antibody against a different region of the OTC protein (**Supplemental Figure 2**) did not improve detection. Immunostaining showed faint basolateral staining in enterocytes of *spf-ash* mice (Figure 2C, **Supplemental Figure 3**); however, in tissue from WT mice, a stronger apical and basolateral staining was observed. A smaller citrulline flux was measured in *spf-ash* mice ($P < 0.001$; Figure 2D) accounting for ~40% of the citrulline produced by the WT mice. Plasma citrulline concentration was also reduced in mutant mice compared with WT controls (25 ± 2 compared with 92 ± 9 μmol/L, respectively; $P < 0.001$). Both arginine flux (320 ± 24 compared with 465 ± 26 μmol · kg⁻¹ · h⁻¹; $P < 0.001$) and plasma concentrations (63 ± 9 compared with 132 ± 3 μmol/L; $P < 0.001$) were lower in *spf-ash* mice than in their littermate WT controls. No differences ($P > 0.45$) in phenylalanine

and tyrosine fluxes and plasma concentrations were observed between the 2 genotypes.

Ex vivo citrulline production. Enteroids derived from both WT and *spf-ash* mice developed at a similar rate and had budding crypt-like structures (**Figure 3**A, B), which showed proliferating cells (Figure 3C, D; **Supplemental Figure 4**). OTC abundance was barely distinguishable in enteroids derived from *spf-ash* mice (**Figure 4**A, B). Immunostaining also showed a faint staining in the mutant enteroids (Figure 4C; **Supplemental Figure 5**), and citrulline production was lower ($P < 0.001$) in enteroids derived from *spf-ash* mice than those derived from WT mice (Figure 4D). The citrulline produced by the mutant enteroids was ~23% of that produced by the WT enteroids.

Discussion

The *spf-ash* mutation is a single nucleotide polymorphism that results in differential splicing of the mRNA, yielding 2 products: one that translates into an enzyme with normal function and another that produces an elongated nonfunctional protein that is immediately degraded (24). Human patients carrying the same nucleotide change have been identified, and this mutation has been associated both with early and late onset of the disease (25).

Despite the reduction in hepatic OTC abundance and only 5–15% residual in vitro enzyme activity compared with WT control mice (26, 27), urea production was not different between the mutant and control mice during feed deprivation. We have shown that B6EiC3Sn *spf-ash* mice are able to sustain ureagenesis despite a large amino acid load (28) and that mutant ICR mice were even more resilient and able to tolerate an unbalanced amino acid load (15). However, a large hepatic capacity to detoxify ammonia exists and thus urea production does not reflect the extent of the OTC deficiency; a high-protein diet (26) or the infusion of an unbalanced amino acid mixture (15, 28) is needed to overwhelm the deficient urea cycle and produce a hyperammonemic phenotype.

Patients carrying a mutation equivalent to the *spf-ash* mice had very low (<3%), but detectable, OTC activity measured in jejunal biopsy samples when compared with control subjects (29). This is similar to the ~6% residual activity measured in the intestine of *spf-ash* mice (27). Here we have shown that the OTC protein in small intestinal tissue was barely detectable; however, citrulline flux in mutant mice was a substantial ~40% of that measured in WT mice. This agrees with previous determinations in both B6EiC3Sn and ICR mutant mice (15). In humans, patients with nonsymptomatic OTC disorder carrying different mutations had circulating citrulline concentrations no different than those in healthy control subjects, although null-OTC patients had very low plasma citrulline (30). Here we have shown a reduced plasma citrulline concentration in mutant mice, which translated into reduced arginine fluxes and plasma concentrations. However, whole-body protein metabolism as measured by phenylalanine-tyrosine did not seem to be affected by the mutation. This highlights the importance of endogenous citrulline-arginine production in the maintenance of arginine homeostasis.

Whole-body citrulline flux includes not only the citrulline produced and released by the gut but also citrulline produced by NO synthase and in the recycling of post-translational modified arginine. However, these last 2 sources account for ~5–8% of the total flux (23), and thus >90% of the citrulline produced is
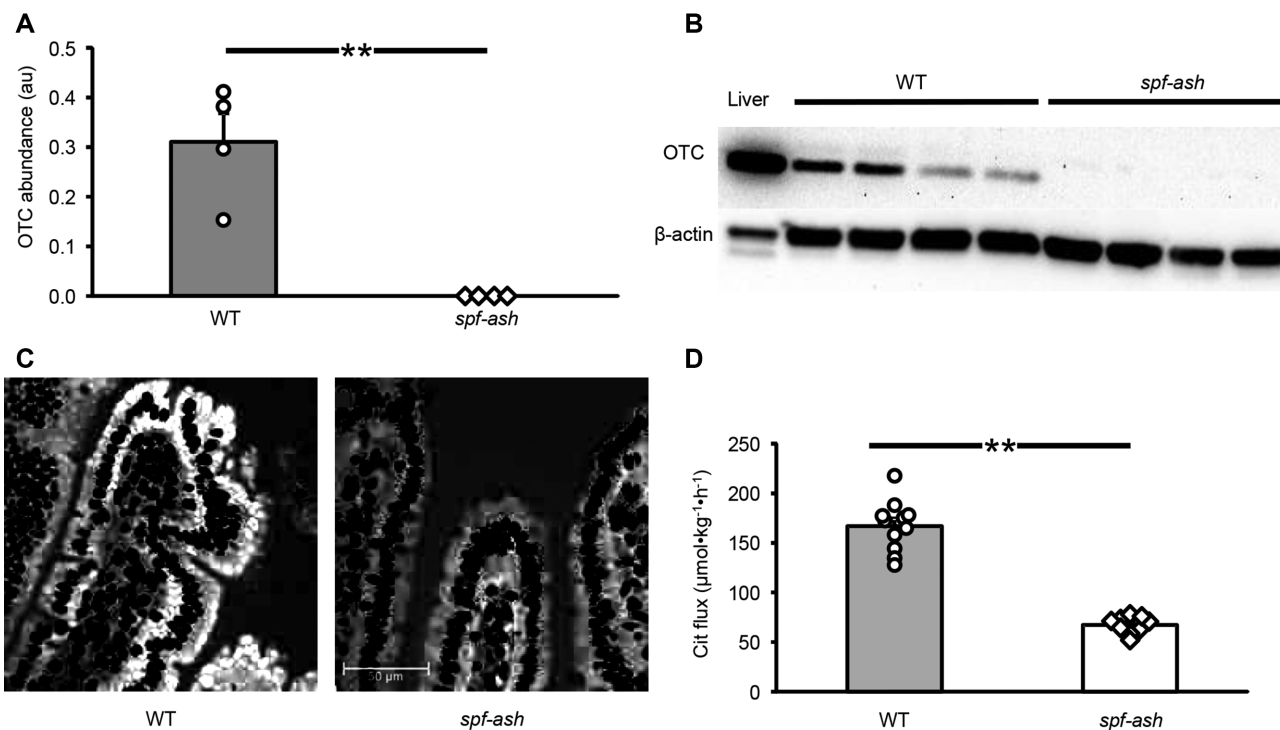
FIGURE 2 Small intestine OTC abundance (A), Western blot (B), immunohistochemistry (C), and citrulline flux (D) in ICR WT and ICR$^{spf\text{-}ash}$ mice. A representative immunochemistry figure, presented here in black and white, has been manipulated to increase the contrast between the nuclei (in black) and the OTC protein (white). (A color figure is available as Supplemental Figure 3.) Values are means ± SEs; $n = 4$ for WT and spf-ash OTC abundance, $n = 11$ for WT, and $n = 9$ for spf-ash for citrulline flux. Individual values are also shown. **$P < 0.001$. au, arbitrary units; Cit, citrulline; ICR, Institute of Cancer Research; OTC, ornithine transcarbamylase; spf-ash, sparse fur and abnormal skin; WT, wild-type.
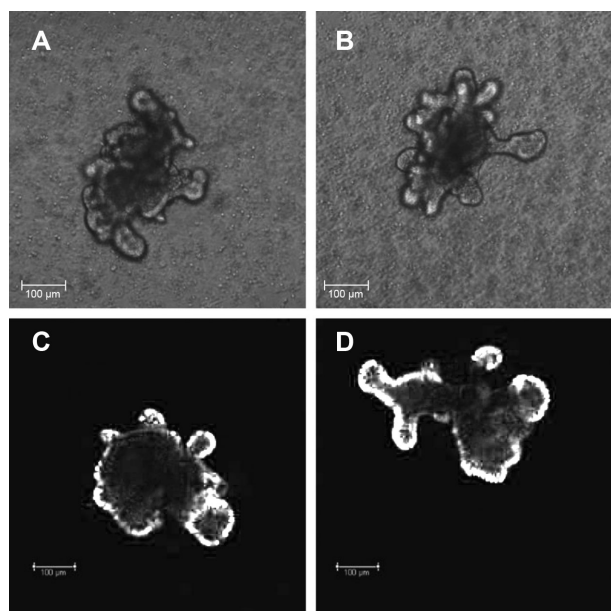

FIGURE 3 Enteroids derived from ICR WT (A, C) and ICR$^{spf\text{-}ash}$ (B, D) mice. Representative enteroid samples are shown using bright-field (A and B) and fluorescence (C and D) microscopy. The fluorescence microscopy figure, presented here in black and white, has been manipulated to increase the contrast of proliferating cells (in white). (A color figure is available as Supplemental Figure 4.) ICR, Institute of Cancer Research; spf-ash, sparse fur and abnormal skin; WT, wild-type.

of enteral origin. For this reason, plasma citrulline concentration has been used as a marker for gut mass and function (31) and functional tests proposed to determine gut function (32). In addition, the lack of correlation between enzyme abundance and in vitro activity with in vivo fluxes (in vivo enzyme activity) shows the metabolic resilience of certain pathways and indicates that metabolic flux control is not only given by enzyme abundances but also by the metabolites involved in the pathway (33).

Enteroids derived from spf-ash mice had a normal development, with budding crypt-like structures that showed proliferating cells. Mutant and WT enteroids recapitulated the in vivo findings: a barely detectable OTC enzyme in enteroids carrying the spf-ash mutation but still able to produce ∼25% of the citrulline synthesized by the WT controls. Thus, enteroids may offer the possibility to study different interventions to increase citrulline ex vivo, before attempting in vivo procedures. This is particularly attractive for the study of different OTC mutations in humans, because in vivo metabolic function does not always correlate with DNA sequence, transcript abundance, or in vitro enzyme activity (34). A limitation of this approach is the need for a biopsy to isolate crypt stem cells; however, the advent of techniques that produce inducible intestinal organoids from pluripotent stem cells (35) will overcome this limitation, and the effect of OTC human mutations on citrulline production can be easily determined.

The advent of novel ex vivo technologies (e.g., "organ on a chip") allows mimicking organ-level complexity by progressively integrating different cell types (including microbes) and studying the system at varying levels of complexity (36, 37). This is particularly attractive for those pathways, such as arginine synthesis, that rely on the cooperation of multiple
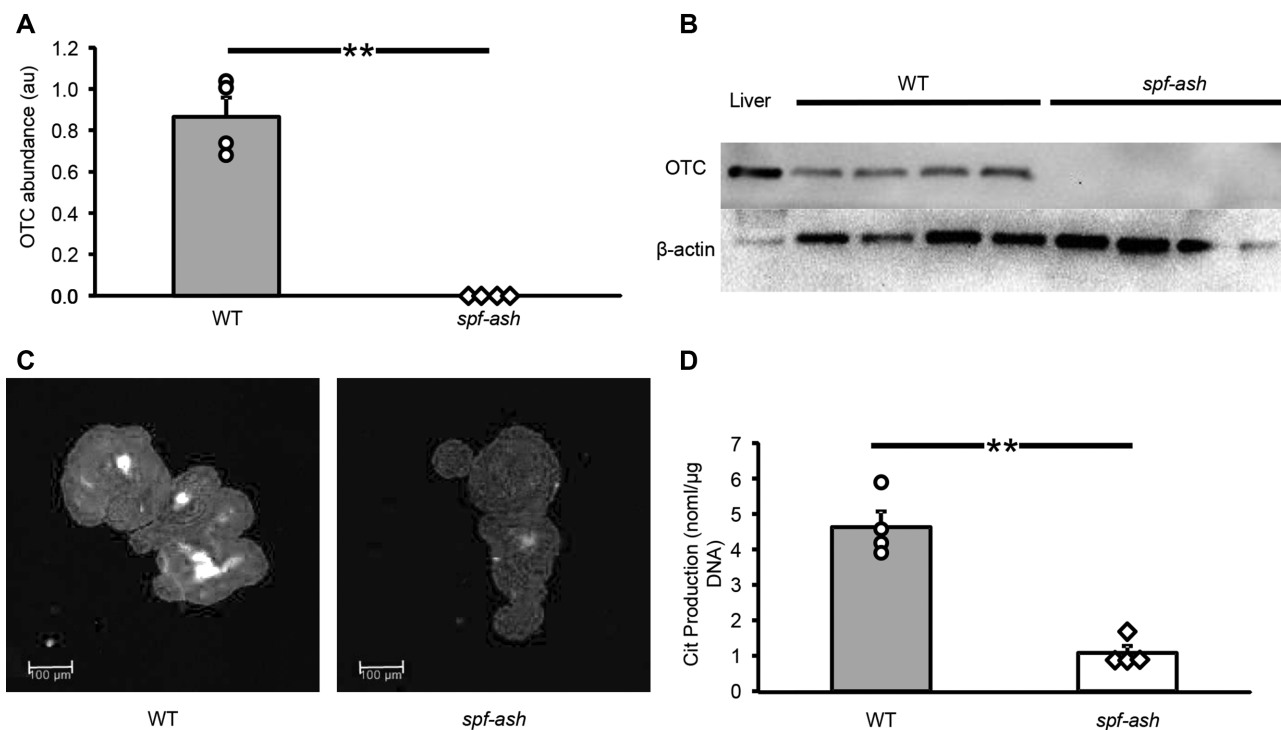
FIGURE 4 OTC abundance (A), Western blot (B), immunohistochemistry (C), and 24-h citrulline production (D) in enteroids derived from the small intestine of ICR WT and ICR$^{spf-ash}$ mice. A representative immunochemistry figure, presented here in black and white, has been manipulated to increase the contrast of the OTC protein (white). (A color figure is available as Supplemental Figure 5.) Values are means ± SEs, $n = 4$ mice. Individual mouse values (mean of 3 wells/mouse) are also shown. **$P < 0.001$. au, arbitrary units; Cit, citrulline; ICR, Institute of Cancer Research; OTC, ornithine transcarbamylase; *spf-ash*, sparse fur and abnormal skin; WT, wild-type.

organs. However, organoids need to be characterized and able to replicate the in vivo metabolic phenotype before they can be incorporated into more complex systems.

In conclusion, enteroids are able to recapitulate in vivo citrulline production and offer the opportunity to study the regulation of citrulline production in a highly manipulable system. In addition, citrulline ex vivo may play the same role as that in vivo as a marker of metabolic function and thus be a valuable variable to monitor in enteroid metabolic studies.

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