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Authors

Eng, Serena J
Nonnecke, Eric B
de Lorimier, Arthur J
et al.

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FOXO inhibition rescues α-defensin expression in human intestinal organoids

Serena J. Eng[a,b], Eric B. Nonnecke[a], Arthur J. de Lorimier[c], Mohamed R. Ali[d], Renée M. Tsolis[a], Charles L. Bevins[a], and Paul Ashwood[a,b,1]

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To mediate critical host–microbe interactions in the human small intestine, Paneth cells constitutively produce abundant levels of α-defensins and other antimicrobials. We report that the expression profile of these antimicrobials is dramatically askew in human small intestinal organoids (enteroids) as compared to that in paired tissue from which they are derived, with a reduction of α-defensins to nearly undetectable levels. Murine enteroids, however, recapitulate the expression profile of Paneth cell α-defensins seen in tissue. WNT/TCF signaling has been found to be instrumental in the regulation of α-defensins, yet in human enteroids exogenous stimulation of WNT signaling appears insufficient to rescue α-defensin expression. By stark contrast, forkhead box O (FOXO) inhibitor AS1842856 induced the expression of α-defensin mRNA in enteroids by >100,000-fold, restoring *DEFA5* and *DEFA6* to levels comparable to those found in primary human tissue. These results newly identify FOXO signaling as a pathway of biological and potentially therapeutic relevance for the regulation of human Paneth cell α-defensins in health and disease.

Paneth cell | stem cell | alpha-defensin | FOXO | enteroid

In addition to providing trophic factors for coresident small intestinal stem cells in the crypts of Lieberkühn (1), Paneth cells maintain homeostasis by both protecting from pathogens and shaping the colonizing microbiota composition via the copious production of antimicrobial proteins and peptides, including α-defensins (2). In humans, α-defensin 5 (DEFA5) and -6 (DEFA6) are abundant secretory products of Paneth cells and provide key nonredundant innate immune functions (2). Multiple lines of evidence suggest that Paneth cell dysfunction and reduced expression of their α-defensins may increase susceptibility to enteric disease (3, 4), including ileal Crohn's disease (5). Moreover, Paneth cell abnormalities have been reported in neurodevelopmental disorders with gastrointestinal comorbidities such as autism spectrum disorder (ASD) (6).

The culture of intestinal stem cells as organoids has transformed the field of gastrointestinal biology. Despite wide acceptance of this method, and the importance of Paneth cell antimicrobial peptides to intestinal homeostasis and barrier defense, studies have relied extensively on lysozyme (LYZ) as the sole marker of human Paneth cells. Few studies have detected α-defensin expression in human intestinal organoids (7–9), and experimental approaches have yet to recapitulate tissue-level expression patterns of these abundant effectors. We sought to determine the utility of patient-derived small intestinal organoids (enteroids) as a model of Paneth cell function.

Materials and Methods

Biopsies from human terminal ileum were obtained (IRB #1400430, "Regulatory Immune Mechanisms and Gastrointestinal Comorbidity in ASD") and cultured as enteroids. This study was approved by the institutional review boards for the State of California and the University of California, Davis. All patients provided informed consent. Detailed materials and methods are provided in *SI Appendix*.

Results and Discussion

The mRNA levels of highly abundant Paneth cell secretory products in human mucosal biopsies (Fig. 1*A*) were consistent with those previously described for small intestinal tissue (10–12), yet the gene expression profile of enteroids (Fig. 1*A*) deviated significantly. Relative quantification of mRNA for each target showed that enteroids derived from paired biopsies retained *LYZ* expression while *DEFA5*, *DEFA6*, intelectin-2 (*ITLN2*), and regenerating family member 3 alpha (*REG3A*) mRNA was present at ~10,000 to ~100,000-fold lower levels than in respective biopsies (Fig. 1*B*).

To investigate whether this loss of α-defensin expression was specific to human enteroid culture, we compared the Paneth cell secretory product profiles of murine small intestinal

Author affiliations: [a]Department of Medical Microbiology and Immunology, School of Medicine, University of California Davis, Davis, CA 95616; [b]Medical Investigation of Neurodevelopmental Disorders Institute, University of California Davis, Sacramento, CA 95817; [c]University of California Davis Medical Center, Department of Pediatrics, Sacramento, CA 95817; and [d]University of California Davis Medical Center, Department of Surgery, Sacramento, CA 95817

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The authors declare no competing interest.

[1]To whom correspondence may be addressed. Email: pashwood@ucdavis.edu.

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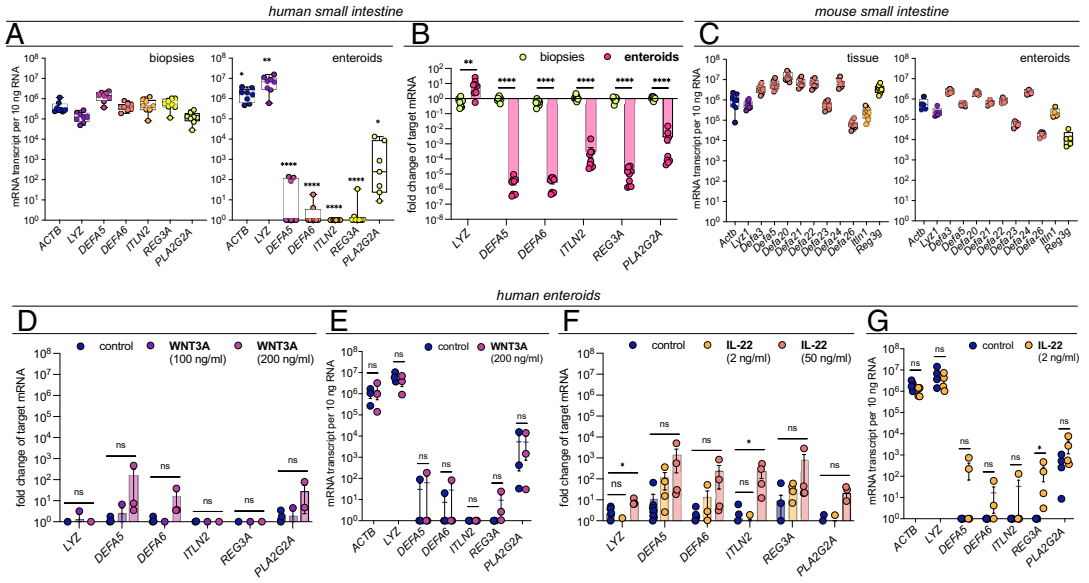

Fig. 1. Human enteroids lose expression of Paneth cell α-defensins. All values are plotted on a logarithmic scale. (*A*) Absolute quantification (biological replicates, n = 8) and (*B*) relative quantification (biological replicates, n = 9) of human Paneth cell secretory effectors in terminal ileum biopsies and respective matched enteroids. (*C*) Expression profile of murine Paneth cell secretory effectors in distal 6-cm small intestine (n = 7 mice) and respective murine enteroids (n = 5 mice) generated from topographically matched tissue specimens from C57BL/6N mice. (*D*) Relative quantification and (*E*) absolute quantification of human Paneth cell secretory effectors in human enteroids (biological replicates, n = 3) either untreated (control) or treated with human recombinant WNT3A (100 ng/mL or 200 ng/mL). (*F*) Relative quantification and (*G*) absolute quantification of human Paneth cell secretory effectors in human enteroids (biological replicates, n = 4) either untreated (control) or treated with human recombinant IL-22 (2 ng/mL or 50 ng/mL). RT-qPCR values for relative quantification are normalized to *ACTB* (β-actin); values for absolute quantification are expressed as quantity of target mRNA transcript per 10 ng of RNA. Statistical analysis: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, ns = nonsignificant. Error bars represent SEM. Fig. 1 *A* and *C* are adapted from ref. 11.

tissue with murine enteroids. We found that murine enteroids recapitulated (within 10-fold) the α-defensin expression profile found in murine ileal tissue (Fig. 1*C*), suggesting that the deficiency in Paneth cell α-defensin production in human enteroids is not due to in vitro culture conditions per se.

The Paneth cell gene expression program is critically dependent on WNT signaling (13), which has been shown to regulate α-defensin expression through TCF (T cell factor) transcription factors (5, 13). While mouse small intestinal Paneth cells directly provide Wnt3 to intestinal stem cells in organoid culture (1), WNT ligands are not produced by human Paneth cells (14) and must be supplemented in human intestinal organoid media. To investigate whether an insufficiency in WNT ligands may explain the absence of α-defensin expression in human enteroids, we supplemented culture media with recombinant human WNT3A (100 or 200 ng/mL) but observed no change in *DEFA5* or *DEFA6* expression (Fig. 1 *D* and *E*). Two WNT target genes, ephrin type-B receptor 3 (*EPHB3*) and leucine rich repeat containing G protein-coupled receptor (*LGR5*), were highly expressed in both WNT3A-treated and untreated enteroids, indicating that WNT signaling was sufficient for transcription of TCF target genes in the culture conditions, yet α-defensin expression was impaired. In addition, we did not find other treatments reported to affect mRNA expression of α-defensins, such as IL-22 at 2 ng/mL (8) or 50 ng/mL to appreciably raise the levels of *DEFA5* or *DEFA6* mRNA (Fig. 1*F*), especially when compared to the expression profile of native tissue (Fig. 1 *A* and *G*).

Knockdown of *Foxo1/3* signaling in a genetic mouse model was found to promote secretory cell differentiation (15), and a cell-permeable inhibitor of FOXO1, AS1842856 (5-amino-7-(cyclohexylamino)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid) induced the secretory lineage of enteroendocrine cells (16). We therefore hypothesized that FOXO could be related to the loss of Paneth cell secretory products in enteroid culture. AS1842856 inhibits the transcriptional activity of FOXO through direct binding to the active (unmodified Ser256) form (17). *FOXO1*, *FOXO3*, and *FOXO4* were confirmed to be expressed in human enteroids (Fig. 2*A*), and expression of canonical FOXO1 target genes glucose-6-phosphatase catalytic subunit (*G6PC*), and phosphoenolpyruvate carboxykinase 1 (*PCK1*), but not FOXO3 target gene catalase (*CAT*) or FOXO3/4 target gene B cell lymphoma 6 (*BCL6*), were reduced after treatment with AS1842856 (Fig. 2*B*).

Remarkably, the treatment of enteroids with FOXO inhibitor AS1842856 at 1 μM increased the expression of *DEFA5* and *DEFA6* >100,000-fold (Fig. 2*C*) to levels not significantly different from small intestinal biopsies (Fig. 2*D*), representing a restorative induction of *DEFA5* and *DEFA6* (Fig. 2 *C–E*) compared to untreated enteroids (Fig. 1 *A* and *B*). Treatment with 500 nM or 1 μM concentrations of AS1842856 were found to mediate this restoration of α-defensins (Fig. 2*F*). AS1842856 treatment of enteroids cultured in differentiation media yielded an induction of α-defensins similar in magnitude (Fig. 2*G*). Fluorescence immunohistochemistry confirmed that while DEFA5 was minimally produced in untreated enteroids (Fig. 2*H*), high levels of DEFA5 peptide were produced in human enteroids treated with AS1842856 (Fig. 2*I*).

Paneth cell products *REG3A*, *PLA2G2A* (group 2 secretory phospholipase A2), and *ITLN2* (12) were also significantly upregulated by AS1842856 (~6,000-fold, ~400-fold, and ~60-fold, respectively), yet not fully restored to tissue levels (Fig. 2 *C–E* and *G*), suggesting that their regulation may involve independent pathways.

A key function ascribed to Paneth cell α-defensins is to shape the composition of the intestinal microbiota (2). While the microbiota can significantly contribute to physiology in the healthy host, perturbations in microbial composition (i.e., dysbiosis) may contribute to the pathogenesis of a wide variety of chronic diseases, including ASD, inflammatory bowel disease (IBD), obesity, diabetes, and cancer (18). Reduced expression of Paneth cell α-defensins has been reported in ileal Crohn's disease and proposed to contribute to the dysbiosis in this disease (5). This novel discovery that treatment with the FOXO inhibitor AS1842856 reconstitutes native-tissue levels of human α-defensins in patient-derived intestinal organoids

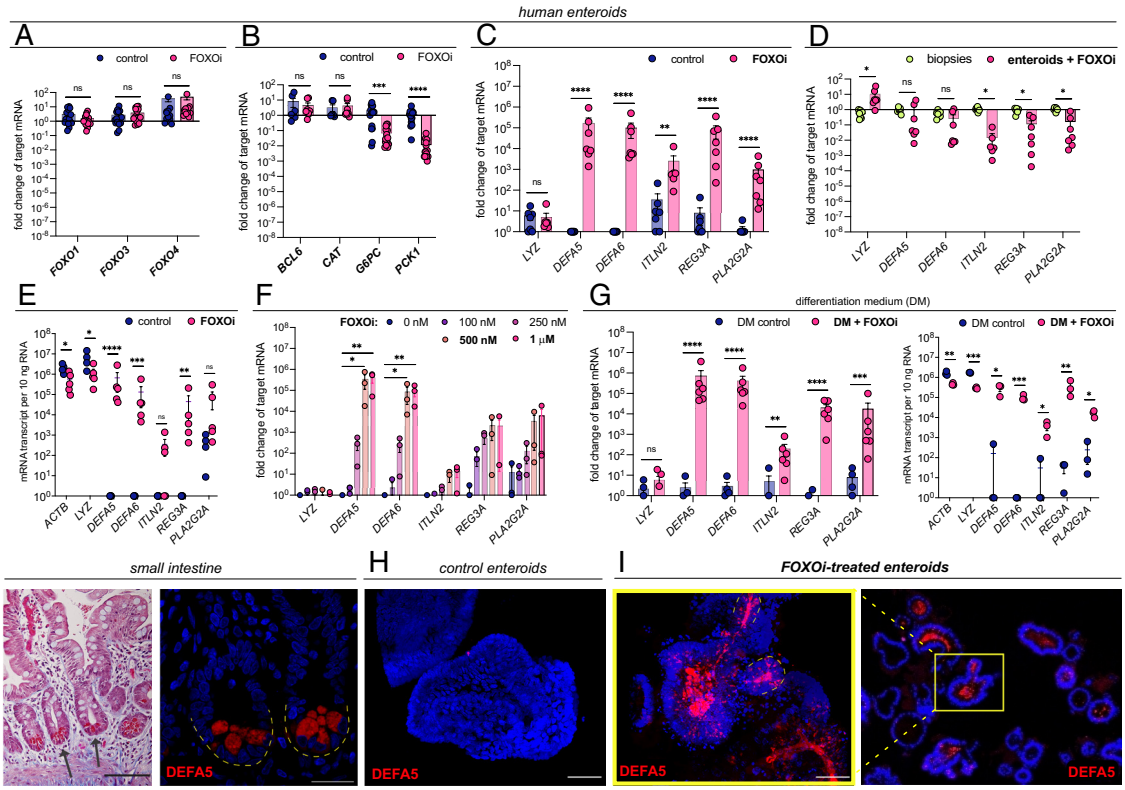

Fig. 2. FOXO inhibition restores α-defensin expression in human enteroids to levels comparable to native tissue. All values are plotted on a logarithmic scale. FOXOi: FOXO inhibitor AS1842856. (*A*) Relative quantification of *FOXO1*, *FOXO3*, and *FOXO4* mRNA in untreated (control) vs. AS1842856-treated (1 μM) human enteroids (n = 16 independent experiments). (*B*) Relative quantification of FOXO target genes in untreated vs. AS1842856-treated (1 μM) human enteroids (n = 7–15 independent experiments). (*C*) Relative quantification (biological replicates, n = 7) of human Paneth cell secretory effectors in untreated vs. AS1842856-treated (1 μM) human enteroids. (*D*) Relative quantification (biological replicates, n = 7) of human Paneth cell secretory effectors in terminal ileum biopsies vs. AS1842856-treated (1 μM) human enteroids. (*E*) Absolute quantification (biological replicates, n = 5) of human Paneth cell secretory effectors in untreated vs. AS1842856-treated (1 μM) human enteroids. (*F*) Relative quantification (biological replicates, n = 3) of human Paneth cell secretory effectors in human enteroids treated with 100 nM to 1 μM of AS1842856. (*G*) Relative quantification (biological replicates, n = 6) and absolute quantification (biological replicates, n = 3) of human Paneth cell secretory effectors in untreated vs. AS1842856-treated (1 μM) human enteroids in differentiation media (DM). (*H*) Human α-defensin 5 (DEFA5) fluorescence immunohistochemistry in untreated vs. (*I*) AS1842856-treated (1 μM) human enteroids. Red: DEFA5; blue: Hoescht 33258 nuclear counterstain. Human jejunal tissue specimens shown at left for reference: Masson's Trichrome staining (arrows indicate base of crypts) and fluorescence immunohistochemistry of DEFA5-positive Paneth cell granules. Scale bars: light microscopy: 50 μm (40×); confocal microscopy: 25 μm (100×). RT-qPCR values for relative quantification are normalized to *ACTB* (β-actin); values for absolute quantification are expressed as quantity of target mRNA transcript per 10 ng of RNA. Statistical analysis: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, ns = nonsignificant. Error bars represent SEM. Fig. 2 *E*, *H*, and *I* are adapted from ref. 11.

suggests that the FOXO signaling axis might prove a valuable therapeutic target to augment Paneth cell function.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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