Original Research

Proteomic profiling of *FBXW7*-mutant serous endometrial cancer cells reveals upregulation of PADI2, a potential therapeutic target

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Abstract

Background: Despite advancements over the past decade revealing molecular aberrations characteristic of endometrial cancer (EC) subtypes, serous ECs remain difficult to treat and associated with poor outcomes. This is due, in part, to the rarity of these tumors within clinical trials and the inability to directly target the most frequent genomic abnormalities. One of the most commonly somatically mutated genes in serous ECs is the tumor suppressor *F-box and WD repeat domain containing 7 (FBXW7)*.

Methods: To identify changes in protein expression associated with *FBXW7* mutation, we clustered regularly interspaced short palindromic repeats (CRISPR)-edited ARK4 *FBXW7* nonmutant serous EC cells to insert recurrent *FBXW7* mutations. We then compared the liquid chromatography tandem mass spectrometry-based proteomic profiles of CRISPR-edited ARK1 and ARK4 serous EC cells to matched parental cells.

Results: Among distinct total and phosphorylated proteins that were significantly differentially expressed in *FBXW7*-mutant cell lines compared to matched parental lines, we identified increased PADI2 (peptidyl arginine deiminase 2) expression in all ARK1 and ARK4 CRISPR-edited *FBXW7*-mutant cell lines. We further confirmed the correlation between *FBXW7* mutation and increased PADI2 expression in a third biological background, JHU-1 endometrioid EC cells. Finally, we established that PADI2 protein is expressed in primary serous endometrial tumors.

Conclusion: Our findings provide novel insight into proteomic changes associated with *FBXW7* mutation in serous ECs and identify PADI2 as a novel potential therapeutic target for these tumors.

Key words

*FBXW7*, mutation, PADI2, proteomics, UCHL1, uterine neoplasms

1 | INTRODUCTION

Although the most common histotype of endometrial cancers (ECs), endometrioid EC, can frequently be effectively treated through hysterectomy, serous EC is a rarer subtype that is often associated with metastasis, recurrence, therapy resistance, and poor outcome.1,2 Serous ECs and other clinically aggressive subtypes exhibit frequent somatic
mutation of the tumor suppressor \textit{FBXW7} (\textit{F-box and WD repeat domain containing 7}; also known as \textit{FB7}, \textit{CDC4}, and \textit{hAGO}). Somatic \textit{FBXW7} mutations occur in 15\%-29\% of serous ECs, 11\%-39\% of uterine carcinomas, 13\%-25\% of clear cell ECs, and 0\%-15\% of endometrioid ECs (reviewed in Ref. [1]). In serous ECs, somatic mutation hotspots occur at \textit{FBXW7} codons 423, 465, 479, and 505.\textsuperscript{4,6}

Research on serous ECs is hindered in part due to the rarity of these tumors and availability of only small numbers of cell lines. An ideal model system to examine the effects of \textit{FBXW7} mutation has been developed through CRISPR editing of ARK1 serous EC cells to insert recurrent \textit{FBXW7} somatic mutations.\textsuperscript{7} Research comparing the levels of a small number of proteins in parental and CRISPR-edited ARK1 cell lines provided the first insights into the direct biochemical effects of \textit{FBXW7} mutations in the context of serous EC: increased phosphorylation of seven cancer-related proteins detected by Western blot.\textsuperscript{7} Equivalent protein changes also occurred in ARK1 and ARK2 cells transiently expressing mutant \textit{FBXW7}.\textsuperscript{7}

Here, we CRISPR-edited ARK4 serous EC cells to insert recurrent \textit{FBXW7} somatic mutations and performed large-scale tandem mass spectrometry-based proteomic profiling on both ARK1 and ARK4 parental and derivative cells. Our findings provide novel insight into the proteomic changes associated with recurrent \textit{FBXW7} mutation in two biologically distinct serous EC cell lines, which include new potential therapeutic targets, most notably PADI2 (peptidyl arginine deiminase 2). We orthogonally validated increased PADI2 protein expression in ARK1 and ARK4 \textit{FBXW7}-mutant cells via Western blot and demonstrated PADI2 protein expression in primary serous endometrial tumors. To further solidify the correlation of increased PADI2 expression to \textit{FBXW7} mutation, we used CRISPR editing to revert the endogenous \textit{FBXW7} c.C1513T (p.R505C) mutation in JHUEM-1 endometrioid EC cells to a wild-type genotype and showed that PADI2 expression was decreased in CRISPR-edited \textit{FBXW7} nonmutant JHUEM-1 cells compared to parental cells.

## MATERIALS AND METHODS

A summary of methods utilized in this manuscript is provided in Figure 1. The research conducted in this study was excluded from IRB Review per 45 CFR 46 and NIH policy for the use of specimens/data.

### 2.1 Cell culture

ARK1 and ARK4 serous EC cell lines were established\textsuperscript{8} and kindly provided by Dr Alessandro A. Santin (Yale University School of Medicine, New Haven, CT). Both lines were maintained in RPMI + 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO\textsubscript{2}. JHUEM-1 endometrioid EC cells were purchased from Riken Bioresource Research Center and maintained in DMEM/HamF12 + 15% FBS at 37°C in a humidified atmosphere with 5% CO\textsubscript{2}. Cells were counted with a Countess Cell Counter (Thermo Fisher Scientific). All cell lines were authenticated by Laragen, Inc using short tandem repeat profiling prior to shipment to the Washington University Genome Engineering and iPSC Center (GEIC). All cell lines were verified mycoplasma-free by GEIC and authenticated again at the time frozen stocks were established.

### 2.2 Generation of CRISPR-edited \textit{FBXW7}-mutated cell lines

ARK4 cells were CRISPR-edited by GEIC to incorporate \textit{FBXW7} c.C1393T (p.R465C), c.G1436A (p.R479Q), and c.C1513T (p.R505C). ARK4 was edited following published methods\textsuperscript{7} with one exception: RNP complexes were assembled by combining 200 pmol of Alt-R gRNA (Integrated DNA Technologies) with 80 pmol of Cas9 protein (California Institute for Quantitative Biosciences) at room temperature for 10 minutes. JHUEM-1 cells were CRISPR-edited by GEIC to remove the endogenous \textit{FBXW7} c.C1513T (p.R505C) mutation following the methods used for ARK4.
ARK1 and ARK4 parental cells lack FBXW7 exonic mutations (verified by Sanger sequencing as described below); ARK1 exhibits FBXW7 copy number loss, and ARK4 has an unknown FBXW7 copy number status. 9 JHUEM-1 parental cells harbor FBXW7 c.C1513T (p.R505C) and exhibit diploid copy number status. 10

### 2.3 Copy number analysis

Because the JHUEM-1 CRISPR-edited clone did not harbor silent blocking modifications intended to prevent recutting during CRISPR modification, GEIC completed copy number analysis using the Hs02590357 cn TaqMan™ Copy Number Assay (Thermo Fisher Scientific) according to the manufacturer's protocol. Assay controls were the human RNase P TaqMan™ Copy Number Assay (4403328, Thermo Fisher Scientific) and GEIC's AN1 female-induced pluripotent stem cells. DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's protocol and data were analyzed using CopyCaller Software (Thermo Fisher Scientific). Results showed matching diploid status of JHUEM-1 parental cells and the CRISPR-edited derivative line (data not shown).

### 2.4 DNA extraction, polymerase chain reaction (PCR) amplification, and Sanger sequencing

DNA was extracted using the Gentra® Puregene® Kit (Qiagen) according to the manufacturer's instructions. FBXW7 primer sequences are available upon request. All coding exons of FBXW7 were sequenced in ARK4 and JHUEM-1 parental and derivative lines to verify CRISPR edits (Figure S1) using PCR conditions, purification, and Sanger sequencing exactly as previously described. 7

### 2.5 Lysate preparation and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

Cells were plated into four 150 mm dishes at a density of 4 x 10^6 cells per dish and scraped into a total of 4 mL urea lysis buffer (Cell Signaling Technology (CST)) the following day. Triplicate lysates were prepared for each cell line and were immediately frozen on dry ice before shipment to CST®. Cellular extracts were sonicated, centrifuged, reduced with dithiothreitol, and alkylated with iodoacetamide. For phosphorylated protein analysis, 500 μg total protein was digested with trypsin, purified over C18 columns (Waters), and used for immobilized metal affinity chromatography (IMAC) enrichment with Fe-NTA magnetic beads (CST®) as previously described. 11 For total protein analysis, 100 μg of each sample was digested with LysC/trypsin, labeled with TMT10plex™ reagent (Thermo Fisher Scientific), bRP fractionated (96 fractions concatenated nonsequentially to 12), and C18 purified as previously described. 12 LC-MS/MS analysis was performed using an Orbitrap-Fusion™ Lumos™ Tribrid™ mass spectrometer (Thermo Fisher Scientific) as previously described. 11,12

For IMAC phosphopeptide analysis, replicate injections were used for each sample and an HCD-MS2 acquisition method was used with the following parameters: method duration 181 minutes, user-defined lock mass 371.10123, Orbitrap resolution 120K, scan range 300-1500 m/z, maximum injection time 50 ms, AGC target 4E5, dynamic exclusion = 1, exclusion duration 30 seconds, mass tolerance ± 10 ppm, max intensity 1E20, min intensity 500, MS2 isolation mode quadrupole, MS2 isolation window 1.6, activation type HCD, collision energy mode fixed, collision energy 27, detector type Orbitrap, Orbitrap resolution 30K, max injection time 54 ms, and AGC target 5E4.

For total proteome analysis, an MS3 acquisition method was used to reduce ion interference and ratio compression with the following parameters: method duration 210 minutes, user-defined lock mass 371.10123, Orbitrap resolution 120K, scan range 350-1400 m/z, maximum injection time 100 ms, AGC target 5E5, dynamic exclusion = 1, exclusion duration 120 seconds, mass tolerance ± 7 ppm, MS2 isolation mode quadrupole, MS2 isolation window 0.4, activation type CID, collision energy mode fixed, collision energy 35, detector type IonTrap, max injection time 10 ms, AGC target 2E4, MS3 isolation mode quadrupole, isolation window 0.7, multi-notch isolation, MS2 isolation window 3 m/z, number of notches = 10, collision energy mode fixed, collision energy 65, detector type Orbitrap, Orbitrap resolution 50K, max injection time 150 ms, and AGC target 2.5E5.

### 2.6 Peptide and protein identification

Mass spectra were evaluated by CST® using SEQUEST and the GFY-Core platform (Harvard University). 13-15 Searches were performed against the 20180718 update of the Uniprot Homo sapiens database with a mass accuracy of ± 50 ppm for precursor ions and 0.02 Da for product ions. Total proteome data were filtered to a 1% peptide-level false discovery rate (FDR) with mass accuracy ± 5 ppm on precursor ions and presence ions; IMAC data were filtered to samples with a phosphorylated residue prior to filtering to a 1% protein-level FDR. All IMAC quantitative results were generated using Skyline16 to extract the integrated peak area of the corresponding peptide assignments. Accuracy of
quantitative data was ensured by manual review in Skyline or in the ion chromatogram files. TMT quantitative results were generated using the GFY-Core platform (Harvard University).

2.7 | Filtering of proteins significantly differentially expressed in FBXW7-mutation knock-in cells

Total proteins were filtered to those significantly ($P < .05$) differentially expressed in FBXW7-mutation knock-in cells compared to matched parental cells with $\geq \pm 2.0$ average fold change. $P$-values were based on a two-tailed $t$ test using the signal:noise for each mutation knock-in cell line versus the matched parental cell line across a minimum of three replicates. Phosphorylated peptides were filtered using these same criteria with additional filters of maximum abundance $> 1\,000\,000$ and percent coefficient of variance ($\%CV$) $\leq 50\%$ for peptides with $\leq 10$ average fold change or $\leq 80\%$ for peptides with $> 10$ average fold change. Unsupervised hierarchical clustering was performed using Partek® Genomics Suite®.

2.8 | Functional annotation of proteins significantly differentially expressed in FBXW7-mutation knock-in cells

Proteins were functionally annotated using g:Profiler$^{17}$ and Ingenuity Pathway Analysis (IPA) (QIAGEN Inc, https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis).

2.9 | Western blot analysis

Protein isolation, quantification, gel electrophoresis, and transfer were performed following published methods$^7$ 1 day after plating $5.5 \times 10^5$ cells per 60-mm dish. Protein was extracted from six primary serous endometrial tumors using a protocol modified from Peña-Llopis and Brugarolas.$^{18}$ Briefly, tissue was homogenized in 10 volumes of RIPA buffer (Thermo Fisher Scientific) containing complete tablet protease inhibitors (Sigma-Aldrich), $0.1\text{-mmol/L NaVO}_4$, and $10\text{-mmol/L NaF}$ (New England BioLabs) while alternating between wet and dry ice. Lysates were centrifuged twice through a QIAshredder column (Qiagen), then cleared using centrifugation.
Proteins were subjected to electrophoresis into Bolt 8% or 10% Bis-Tris Gels (Thermo Fisher Scientific) and wet-transferred to PVDF membranes (Bio-Rad Laboratories). Blots were blocked in Tris-buffered saline containing 1% Tween 20 (TBST) + 5% milk and incubated in the following antibodies from CST	extsuperscript{TM} according to their suggested protocols: PADI2 (#97647), UCHL1 (#13179), P-MARCKS (#8722), MARCKS (#5607), NDRG1 (#9485), TGM2 (#3557), and EPLIN (#50311). As a loading control, blots were incubated in β-Actin antibody (A2228; Sigma-Aldrich) overnight at 4°C at a dilution of 1:5000 in TBST + 5% milk. Secondary antibodies were Cell Signaling Western ECL Substrate (Bio-Rad Laboratories) prior to film exposure (Carestream Health, Inc or Agfa Healthcare NV) and development using a film processor (Konica Minolta, Ramsey, NJ). To enable probing with multiple antibodies, blots were incubated in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific). All Western blots are representative of a minimum of three lysate replicates.

3 | RESULTS

We CRISPR-edited the ARK4 serous EC cell line to generate three derivative cell lines, each harboring one recurrent FBXW7 mutation (p.R465C, p.R479Q, or p.R505C) (Figure S1). To identify proteomic changes associated with recurrent FBXW7 mutations in serous ECs, we performed LC-MS/MS proteomic profiling for ARK1 and ARK4 FBXW7-mutant cell lines in triplicate and compared phosphorylated and total protein expression of FBXW7-mutated cell lines to that of the matched FBXW7 nonmutated parental lines.

Following stringent data filtration (see Methods and Figure 1), 710 and 963 phosphorylated peptides were significantly differentially expressed in ARK1 and ARK4 FBXW7-mutated cell lines, respectively, compared to matched parental lines (Figures 2-4, Tables S1-S6). Phosphopeptides corresponding to 127 proteins were significantly differentially expressed in at least two FBXW7-mutant ARK1 cells compared to ARK1 parental cells (Figure 3) and mapped most significantly (P < 2.7 × 10^{-6}) to the Gene Ontology (GO) terms cytoskeletal binding, cadherin binding, and cell adhesion molecule binding; the only significant (P < 8.5 × 10^{-5}) KEGG pathway was tight junction (Table S7). Phosphopeptides corresponding to 185 proteins were significantly differentially expressed in at least two FBXW7-mutant ARK4 cell lines compared to ARK4 parental cells (Figure 4) and mapped most significantly (P < 2.7 × 10^{-10}) to the GO terms protein binding, cytoskeletal protein binding, and actin binding; the

![Venn diagram of phosphorylated proteins](image-url)
only significant ($P < 2.3 \times 10^{-6}$) KEGG pathway was adherens junction (Table S8). Phosphopeptides corresponding to three proteins (FYTTD1 (forty-two-three-domain containing 1), TNKS1BP1 (tankyrase 1 binding protein 1), and AHNAK (AHNAK nucleoprotein)) were significantly differentially expressed in all ARK1 and ARK4 FBXW7-mutated cell lines.

In total proteome analyses, 225 and 104 proteins were significantly differentially expressed among ARK1 and ARK4 FBXW7-mutated cell lines, respectively, compared to matched parental lines (Figures 5-6, Tables S9-14). Sixty-six proteins were significantly differentially expressed in at least two ARK1 FBXW7-mutant lines (Figure 6A) and significantly ($P < .002$) mapped to the extracellular exosome GO term, while 24 proteins were significantly differentially expressed in at least two ARK4 FBXW7-mutant lines, compared to parental lines (Figure 6B, Tables S15-16).

We aimed to identify proteins that were changed in both biologically distinct serous EC cell lines (ARK1 and ARK4). A sum of 110 unique total and phosphorylated proteins were differentially expressed in at least one ARK1 and one ARK4 CRISPR-edited FBXW7-mutant cell line compared to the matched parental cell line (Figure 7A). GO terms most significantly ($P < 3.3 \times 10^{-6}$) associated with these proteins were protein binding, cadherin binding, cell adhesion molecule binding, and cytoskeletal protein binding (Figure 7D, Table S17). Networks generated using Ingenuity Pathway Analysis indicated that these 110 proteins act in pathways known to contribute to serous ECs, including the mitogen-activated protein kinase (MAPK), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and tumor protein 53 (p53) pathways (Figure S2). Of these 110 differentially expressed proteins, PADI2 was conspicuous in that it exhibited increased expression (2.3- to 7.6-fold change, Figure 8C) across all FBXW7-mutant lines compared to respective parental lines (Figure 7A) and is potentially directly druggable.

We used Western blot analyses to orthogonally validate the increased protein expression of PADI2 in all ARK1 and ARK4 FBXW7-mutant cell lines as well as the expression changes of five additional proteins (UCHL1 (ubiquitin C-terminal hydrolase L1), MARCKS (myristoylated alanine-rich protein kinase C substrate), NDRG1 (N-myc downstream regulated 1), TGM2 (transglutaminase 2), and LIMA1 (LIM domain and actin binding 1)) that were differentially expressed across different ARK1 and ARK4 derivative lines and for which antibodies were readily available (Figure 8A-C). To further confirm the correlation between FBXW7 mutation and PADI2 protein expression, we reverted the endogenous FBXW7 c.C1513T (p.R505C) mutation to wild-type in JHUEM-1 endometrioid EC cells. Compared to parental cells, CRISPR-edited FBXW7 wild-type JHUEM-1 cells exhibited decreased PADI2 expression (Figure 8D), consistent with the increased PADI2 expression observed in
Unsupervised hierarchical clustering of normalized signal:noise for total proteome peptides significantly (P < .05) differentially expressed in CRISPR-edited FBXW7-mutated cells compared to respective FBXW7 nonmutant parental cells in replicate lysates prepared for each cell line. ARK4 replicate 2 was run twice (to fill extra 10-plex runs; both values for replicate 2 were used in final calculations). Fold change is not shown on this figure and was calculated within replicates, comparing mutant line expression to parental. Proteins significantly differentially expressed in all three ARK1 or ARK4 FBXW7-mutant lines compared to respective parental lines are boxed. Protein names and fold change values are provided in Figure 6 and Tables S9-S14. Arrows indicate PADI2, which was significantly differentially expressed in all three ARK1 and ARK4 FBXW7-mutant lines compared to respective parental controls.

Venn diagrams of total proteome proteins significantly (P < .05) differently expressed (≥±2.0 average fold change) in (A) CRISPR-edited ARK1 FBXW7-mutant cell lines and (B) CRISPR-edited ARK4 FBXW7-mutant cell lines compared to matched parental lines.
Serous ECs are challenging tumors associated with metastasis, recurrence, and poor outcome.\(^1,2\) Research over the last decade has revealed molecular aberrations that occur in these aggressive tumors and ongoing efforts are aimed at determining the functional consequences and clinical utility of these abnormalities. Given the high frequency of somatic \(FBXW7\) mutation in serous ECs (range 15\%-29\%; reviewed in Ref. \(^3\)), we sought to identify potentially druggable protein changes associated with three recurrent somatic \(FBXW7\) mutations (p.R465C, p.R479Q, and p.R505C), which account for 34\% of all \(FBXW7\) mutations reported in serous endometrial tumors.\(^5-6,19\)

Through LC-MS/MS-based analyses and Western blot validation, we identified upregulated PADI2 protein in conjunction with all \(FBXW7\) hotspot mutations. To our knowledge, this is the first report of PADI2 protein expression in serous ECs and the first association between increased PADI2 protein and \(FBXW7\) mutations in any tumor type. Furthermore, our correlation of increased PADI2 expression to an endogenous \(FBXW7\) mutation (p.R505C) in JHUEM-1, a grade 2, microsatellite instable (inferred by Ref. \(^10\)) endometrioid EC cell line, indicates that our results may be generalizable across EC histological and molecular subtypes.

Herein, we demonstrated PADI2 protein expression in six primary endometrial tumors but lacked appropriate matched normal endometrium samples for comparison. However, the expression of PADI2 has been characterized in non-uterine cancers. Compared to normal tissue, PADI2 protein is increased in breast, cervical, colon, liver, lung, ovarian serous, thyroid,\(^20\) and prostate cancers.\(^21\) PADI2 mRNA expression is upregulated in tamoxifen-resistant\(^22\) and HER2/ ERBB2 + breast cancers.\(^23\) In contrast, in colon cancer, PADI2 mRNA and protein are decreased\(^24,25\) and low mRNA correlates with poor prognosis.\(^24\)

PADI2 catalyzes citrullination, whereby peptidylarginine is converted into peptidyl-citrulline. Substrates of
PADI2 include histones, vimentin, β- and γ-actins, myelin basic protein, and β-catenin. In preclinical models of breast cancer, prostate cancer, gastric cancer, and glioblastoma, PADI2 inhibition or depletion decreased growth, migration, and extracellular vesicle release, and was synergistic with docetaxel and bortezomib in inducing apoptosis in tamoxifen-resistant breast cancer and bone marrow mesenchymal stem cells, respectively. Additionally, overexpression of PADI2 in a transgenic mouse model caused the development of spontaneous skin neoplasms. In contrast, in colon cancer cells, overexpression of PADI2 decreased growth while deletion of
PADI2 increased growth, reduced cell contact inhibition, and reduced sensitivity to nitazoxanide. We anticipate future research into the functional effects of PADI2 dysregulation in ECs.

Our results provided insight into the global proteomic changes associated with FBXW7 mutations in serous EC cells. Our stringent filtering criteria led to the identification of proteins that were reproducibly altered across triplicate lysates (Figures 2 and 5) and included 21 proteins identified as known or potential FBXW7 substrates differentially expressed in HCT116 FBXW7 knockout colorectal cancer cells compared to wild-type cells (Table S19). Among these proteins was cyclin E1, which validates a previous report of increased cyclin E1 in proteins was cyclin E1, which validates a previous report of metrioid EC patients. Immunohistochemical expression of dation in breast cancer cells. This raises the possibility that same report. Our results provide the additional novel insight into the global proteomic changes associated with mutations in serous EC cancers. Our stringent filtering criteria led to the identification of proteins altered by FBXW7 changes associated with mutations, FBXW7 would occur with alternate restrictions. Data are not publicly available due to privacy and ethical considerations. The data we provide herein is a valuable compendium of whole proteome, which will provide a foundation for future research. Combining our data with recent literature implicating the function of FBXW7, will be important. Finally, our work restricts to the identification of proteins altered by FBXW7 mutation; however, the results provided herein combined with recent literature implicating the function of PADI2 in other tumor types and of UCHL1 in serous ECs, as well as the potential to directly drug both proteins provides significant impetus for future research.

CRISPR-edited ARK17 and ARK4 FBXW7-mutant derivative serous EC cell lines are important resources to aid the study of FBXW7 mutations in this rare tumor type. By comparing the proteomic profiles of FBXW7-mutant derivative lines to matched parental lines, we identified changes that occur across cells harboring distinct FBXW7 mutations (p.R465C, p.R479Q, and p.R505C) and across cells with different genomic backgrounds (ARK1 and ARK4). We orthogonally validated expression changes in a number of proteins, including increased PADI2 in all FBXW7-mutant cell lines. We further confirmed increased PADI2 protein in FBXW7 p.R505C-mutant JHUEM-1 endometrioid EC cells compared to CRISPR-edited FBXW7 nonmutant JHUEM-1 cells. Finally, we are the first to demonstrate PADI2 protein expression in primary serous endometrial tumors. These results lay a solid foundation for future research to investigate the therapeutic potential of PADI2 in serous and other FBXW7-mutated ECs.

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CONFLICT OF INTEREST
Mary Ellen Urick has no conflict of interest. In the interest of full disclosure, Daphne W. Bell receives royalty income from US patent No. 7,294,468 for work unrelated to the current study.

AUTHOR CONTRIBUTIONS
Daphne W. Bell co-conceptualized and supervised the study and provided critical edits to the article. Mary Ellen Urick co-conceptualized the study, performed all experiments not designated to a contracting company in the methods, completed formal analysis, and data curation, and wrote the original draft of the article.

DATA AVAILABILITY STATEMENT
Additional data supporting our study conclusion are presented as supplementary material and are available from the corresponding author upon reasonable request. The data are not publicly available due to privacy and ethical restrictions.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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