Journal of proteome research

pubs.acs.org/jpr

Article

Peptide Derived from AHNAK Inhibits Cell Migration and Proliferation in Hirschsprung's Disease by Targeting the ERK1/2 Pathway

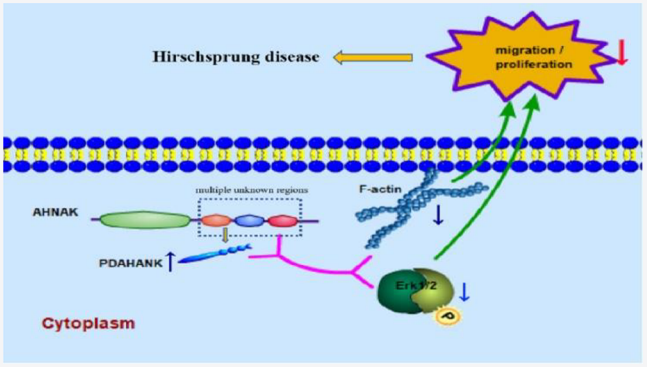
Yuhan Li, Xiurui Lv, Huan Chen, Zhengke Zhi, Zhonghong Wei, Binyu Wang, LingLing Zhou, Hongxing Li,* and Weibing Tang*

Cite This: *J. Proteome Res.* 2021, 20, 2308−2318

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Hirschsprung's disease (HSCR) is characterized by the lack of ganglion cells in the distal part of the digestive tract. It occurs due to migration disorders of enteric neural crest cells (ENCCs) from 5 to 12 weeks of embryonic development. More and more studies show that HSCR is a result of the interaction of multiple genes and the microenvironments, but its specific pathogenesis has not been fully elucidated. Studies have confirmed that many substances in the intestinal microenvironment, such as laminin and β1-integrin, play a vital regulatory role in cell growth and disease progression. In addition to these high-molecular-weight proteins, research on endogenous polypeptides derived from these proteins has been increasing in recent years. However, it is unclear whether these endogenous peptides have effects on the migration of ENCCs and thus participate in the occurrence of HSCR. Previously, our research group found that compared with the normal intestinal tissue, the expression of AHNAK protein in the stenosed intestinal tissue of HSCR patients was significantly upregulated, and overexpression of AHNAK could inhibit cell migration and proliferation. In this study, endogenous peptides were extracted from the normal control intestinal tissue and the stenosed HSCR intestinal tissue. The endogenous polypeptide expression profile was analyzed by liquid chromatography−mass spectrometry, and multiple peptides derived from AHNAK protein were found. We selected one of them, "EGPEVDVNLPK", for research. Because there is no uniform naming system, this peptide is temporarily named PDAHNAK (peptide derived from AHNAK). This project aims to clarify the potential role of PDAHNAK in the development of HSCR and to further understand its relationship with its precursor protein AHNAK and how they contribute to the development of HSCR.



KEYWORDS: *Hirschsprung's disease, endogenous polypeptide, PDAHNAK, AHNAK, proliferation, migration*

1. INTRODUCTION

Hirschsprung's disease (HSCR) is a common gastrointestinal malformation characterized by the absence of ganglia in the intestinal wall of the distal intestine.[1,2] The latest research shows that many coding genes are related to the occurrence of HSCR,[3] such as RET, GDNF, EDNRB, and SOX10. However, these genetic mutations can only explain no more than 30% of HSCR cases, which indicates that the mechanism behind the occurrence of HSCR requires further exploration.

In recent years, with the in-depth study of the pathogenesis of HSCR, it was found that changes in the microenvironment of the intestinal wall will affect the migration and development of intestinal nerve cells. Cell migration is largely affected by the extracellular matrix and cytoskeleton. Many extracellular matrix proteins are involved in the migration of enteric neural crest cells (ENCCs). Akbareian[4] and others found that tenascin-C is dynamically expressed during the development of ENCCs, and ENCCs can also secrete tenascin-C. In the mouse neural tube culture model, tenascin-C could promote the migration of ENCCs. Another study found that altering the expression of laminin LAMA1 in the extracellular matrix may impair the migration of ENCC and lead to the occurrence of HSCR.[5] In addition, the study also found that β1-integrin as a transmembrane integrin is closely related to the function of focal adhesions, and its absence will seriously hinder the migration of ENCCs in the mouse colon.[6] AHNAK, also known as desmoyokin, is a macromolecular protein that was originally

Received: October 21, 2020
Published: April 15, 2021

identified as a desmosomal junction protein.[7] It can regulate Ca^{2+} channels and actin to form a cytoskeleton, which in turn affects cell migration.[8] There are also studies showing that AHANK can affect cell migration ability by reducing the activity of ERK1/2.[9] Previous research by our research group found that the expression of AHNAK in the stenosed HSCR intestinal tissue was significantly higher than that of the normal control intestinal tissue (Figure S1). *In vitro* functional experiments showed that overexpression of AHNAK can inhibit the proliferation and migration of nerve-related cells.

Recently, more and more studies have found that not only high-molecular-weight proteins but also some relatively simple structures, consisting of only 2−100 amino acid endogenous peptides, play an important role in both physiological and pathological processes, and due to the small molecular weight, they can pass through various physiological barriers easily. For example, erythropoietin-derived polypeptides can inhibit the differentiation of mouse adipocytes and the secretion of inflammatory cytokines.[10] After intraperitoneal injection of erythropoietin-derived polypeptides in mice, their insulin resistance improved significantly. Polypeptide SPAR can regulate multiple cellular processes, affecting muscle regeneration and injury repair.[11] However, little is known about whether such endogenous peptides affect the proliferation and migration of ENCCs and participate in the occurrence of HSCR.

Our study examined the potential role of endogenous peptides from the intestinal wall microenvironment in the occurrence of HSCR. First, using Covaris non-contact ultrasonic ultrafiltration, endogenous peptides were extracted from the normal control intestinal tissue and the stenosed intestinal tissue from HSCR patients. Then, the endogenous polypeptide expression profile was analyzed by liquid chromatography/mass spectrometry. According to the sequencing results (Figure S2), compared with the control group, 23 peptides derived from AHNAK exhibited significantly increased expression in HSCR stenosis tissues. Four peptides with $p < 0.002$ were selected, and their biological characteristics were analyzed through the biochemical analysis website ProtParam (https://web.expasy.org/protparam/). We found that among these peptides derived from AHNAK, the peptide sequence "EGPEVDVNLPK" not only was significantly upregulated but also had more stable physical and chemical properties (Figure S2), so we chose this peptide for further study. Because there is no unified naming convention, we temporarily refer to this endogenous peptide derived from AHNAK as PDAHNAK (peptide derived from AHNAK).

Then, through enzyme-linked immunosorbent assay (ELISA) experiments, it was further confirmed that the expression of PDAHNAK in the stenosed segment was significantly increased compared to the normal control and the dilated segment from HSCR patients. Also, *in vitro* and in vivo functional experiments showed that the endogenous polypeptide PDAHNAK could inhibit the proliferation and migration of nerve-related cells. In order to further understand the mechanism of the PDAHNAK function, we found that PDAHNAK can inhibit the phosphorylation of ERK1/2. A functional synergy between PDAHNAK and its precursor protein AHNAK was also discovered. This study initially explored the potential role of endogenous peptide PDAHNAK in the occurrence of HSCR and the functional relationship between the peptide and its precursor protein, providing new insights into understanding the pathogenesis of HSCR.

2. MATERIALS AND METHODS

2.1. Clinical Tissue Samples, Animal Maintenance, and the Ethics Statement

In this study, 48 human colon tissue samples were adopted including 24 from HSCR patients confirmed by histopathological diagnosis and 24 matched normal tissues. The normal tissues were derived from patients diagnosed without HSCR or other enteric neural malformations in Children's Hospital of Nanjing Medical University (collected from 2009 to 2018 after surgery). All human colon tissue samples were frozen at a liquid nitrogen tank at once after surgery. Also, each patient has provided written informed consent. The clinical information of patients is listed in Table 1. The wild-type zebrafish strain used in this study is Tübingen (Tu) strain zebrafish, purchased from the Model Animal Center of Nanjing University (Nanjing, China). The cultivation conditions of zebrafish are as follows: ambient temperature, 28 ± 0.5 °C; daily light 14 h; avoid light for 10 h. Zebrafish are fed twice a day. In this experiment, zebrafish embryos were obtained through natural mating. The Institutional Ethics Committee of Nanjing Medical University authorized and supervised this research.

Table 1. Clinical Characteristics of Study Population

variable	control (n = 24)	HSCR (n = 24)	P
age (days, mean, SE)	159.30 (35.95)	124.60 (6.856)	0.35[a]
	Sex (%)		
male	15 (62.50)	20(83.33)	0.10[b]
female	9 (37.50)	4 (16.67)	

[a]Student's t-test. [b]Two-sided chi-squared test.

2.2. Cell Lines and Culture

Human 293T and SK-N-BE2 cell lines were purchased from American Type Culture Collection (ATCC, Manassas VA, USA). The cell culture medium was prepared using Dulbecco's modified Eagle's medium (DMEM) (Hyclone, UT, USA), 10% fetal bovine serum, and 100 U/mL penicillin. The cells were cultured under the conditions of 37 °C and 5% carbon dioxide.

2.3. Cell Transfection

"EGPEVDVNLPK" was synthesized by Nanjing Binogy Biology Technology Co., Ltd. The AHNAK overexpressing plasmids were purchased from GeneChem (Shanghai, China). Here, the peptide powder is diluted with ddH_2O to a concentration of 100 μg/mL and stored at −20 °C. The peptide sequence synthesized *in vitro* is directly incubated with the cells to increase the expression of PDAHNAK in the cells. The plasmid AHNAK was added to the cell culture medium together with Lipofectamine3000 Reagent (Invitrogen, CA, USA) according to the experimental manual to achieve the purpose of overexpressing the protein AHANK.

2.4. Cell Proliferation Assay

Cells were cultured on 96-well plates with serum-free DMEM overnight. After 1 h incubation of the CCK-8 reagent (10 μL/well, Dojindo, Japan), we measured the absorbance at the wavelength of 450 nm using a TECAN infinite M200 Multimode microplate reader (Tecan, Mechelen, Belgium). The ability of cell growth was also assessed by the 5-ethynyl-20-deoxyuridine (EdU; Roche) (Ribobio, Guangzhou, China) assay. Each assay was repeated three times independently.

https://doi.org/10.1021/acs.jproteome.0c00811
J. Proteome Res. 2021, 20, 2308−2318

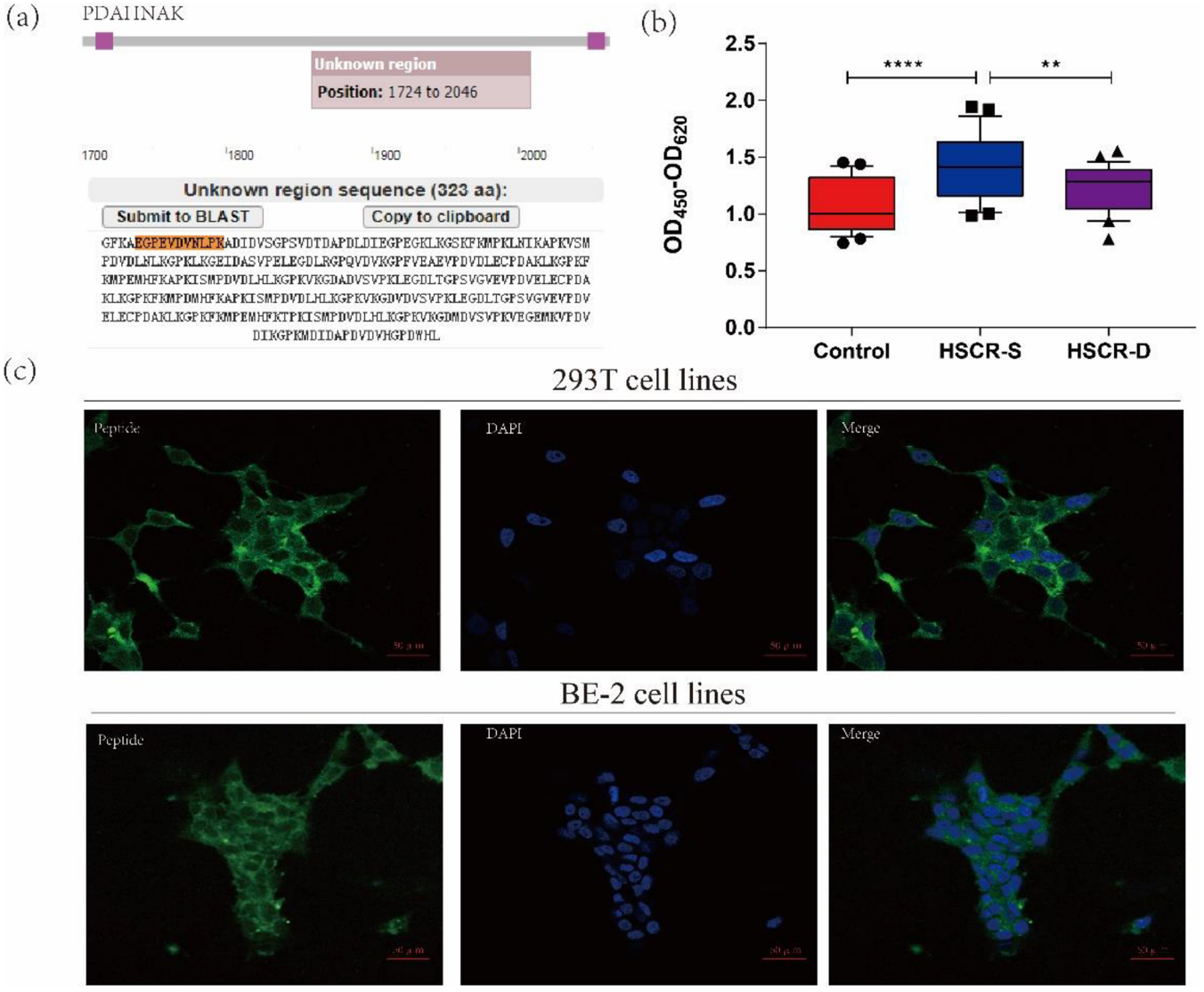


Figure 1. Overexpression of PDAHNAK in HSCR. (a) The sequence was found to be derived from multiple unknown regions of the precursor protein AHNAK (one of which is shown). (b) PDAHNAK expression was assessed in 24 pairs of HSCR and the control using ELISA. PDAHNAK was significantly increased in the HSCR-structured tissue compared to the HSCR-dilated and control with about a 2−4-fold change. (c) It is verified by immunofluorescence that PDAHNAK after 24 h incubation can easily enter into 293T or BE-2 cell lines by diffusion or endocytosis. Scale bar, 50 μm.

2.5. Cell Transwell Assays

We placed 600 μL of the serum medium into the lower chamber. Then, transfected cells were mixed with no serum medium, and about 100 μL (1×10^6 cells/mL) of cells was added into the upper chamber. After 24 h, the cells were fixed and stained. PBS was applied to wash the bottom of the chamber. Finally, a light microscope was used to capture pictures under 20× magnification. Each assay was repeated three times independently.

2.6. Wound Healing Assay

We seeded the 293T and SK-N-BE2 cells on six-well plates. After cells reached 80−90% confluence, a 100 μL pipette tip was used to draw a straight wound on the confluent monolayer. The cells were cultured with the serum-free medium and photographed using an inverted microscope 24 and 36 h later. Each assay was repeated three times independently.

2.7. Cell Cycle and Apoptosis Assays

The assays were conducted and analyzed with a flow cytometer (FACScan; BD Biosciences, USA) equipped with Cell Quest software (BD Biosciences). In general, the cells were trypsinized from a six-well plate and washed twice with PBS and then fixed in 70% cold ethanol and stored at 20 °C prepared for cell cycle analysis. For the cell apoptosis, the cells were washed twice with PBS and then stained with an Annexin V-FITC/Propidium Iodide Kit (BD Biopharmingen, NJ, USA). All assays were repeated three times independently.

2.8. Peptide Extraction and ELISA

The bowel tissues of HSCR and controls were lysed to extract the total proteins by acetic acid (0.25%) with the protease inhibitor. Next, the peptides were extracted from the total proteins with a 30kda Ultrafiltration tube (1.5 mL, Millipore). According to the ELISA kit (Quantikine; R&D Systems) provided by the reagent supplier, we carried out the concentration detection of PDAHNAK. To put it simply, first, dilute the antigen (peptide) to 5 μg/mL in carbonate buffer;

https://doi.org/10.1021/acs.jproteome.0c00811
J. Proteome Res. 2021, 20, 2308−2318

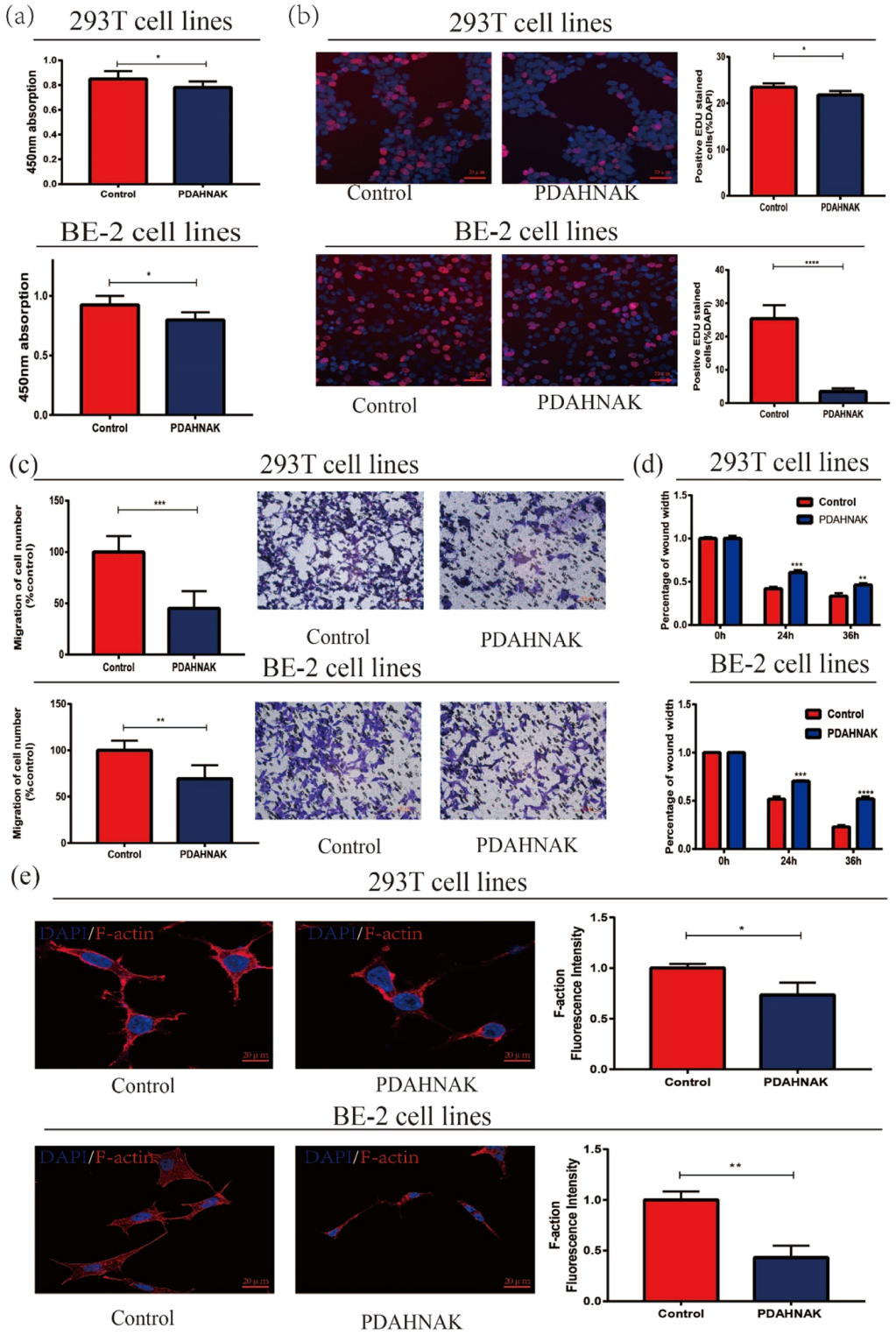


Figure 2. PDAHNAK regulated cell proliferation and migration *in vitro*, and PDAHNAK affected cell cytoskeleton. (a,b) CCK8 assay and EdU assay showed that the upregulation of PDAHNAK inhibited 293T and BE-2 cell proliferation. Scale bar, 50 μm. (c) Transwell assay was used to observe the effects of overexpressed PDAHNAK on cell migration in human 293T and BE-2 cells. Scale bar, 100 μm. (d) Wound healing assay was performed as described in the Materials and Methods section. (e) F-actin cytoskeleton was visualized with rhodamine phalloidin staining in human 293T and BE-2 cells. Scale bar, 20 μm.

then, add 100 μL of the antigen to each well on the ELISA plate and incubate overnight at 4 °C; flick the contents into the sink and wash twice with the washing buffer; next, add 200 μL of the blocking buffer to each well and incubate for 2 h at room temperature; then, discard the blocking buffer and wash three times with the washing buffer; add 100 μL of the diluted primary antibody to each well and incubate for 30−60 min at room temperature; then, wash three times with the washing solution; add 100 μL of HRP-labeled goat anti-rabbit IgG (1:5000, diluted in the blocking buffer) to each well, and incubate at 37

https://doi.org/10.1021/acs.jproteome.0c00811
J. Proteome Res. 2021, 20, 2308−2318

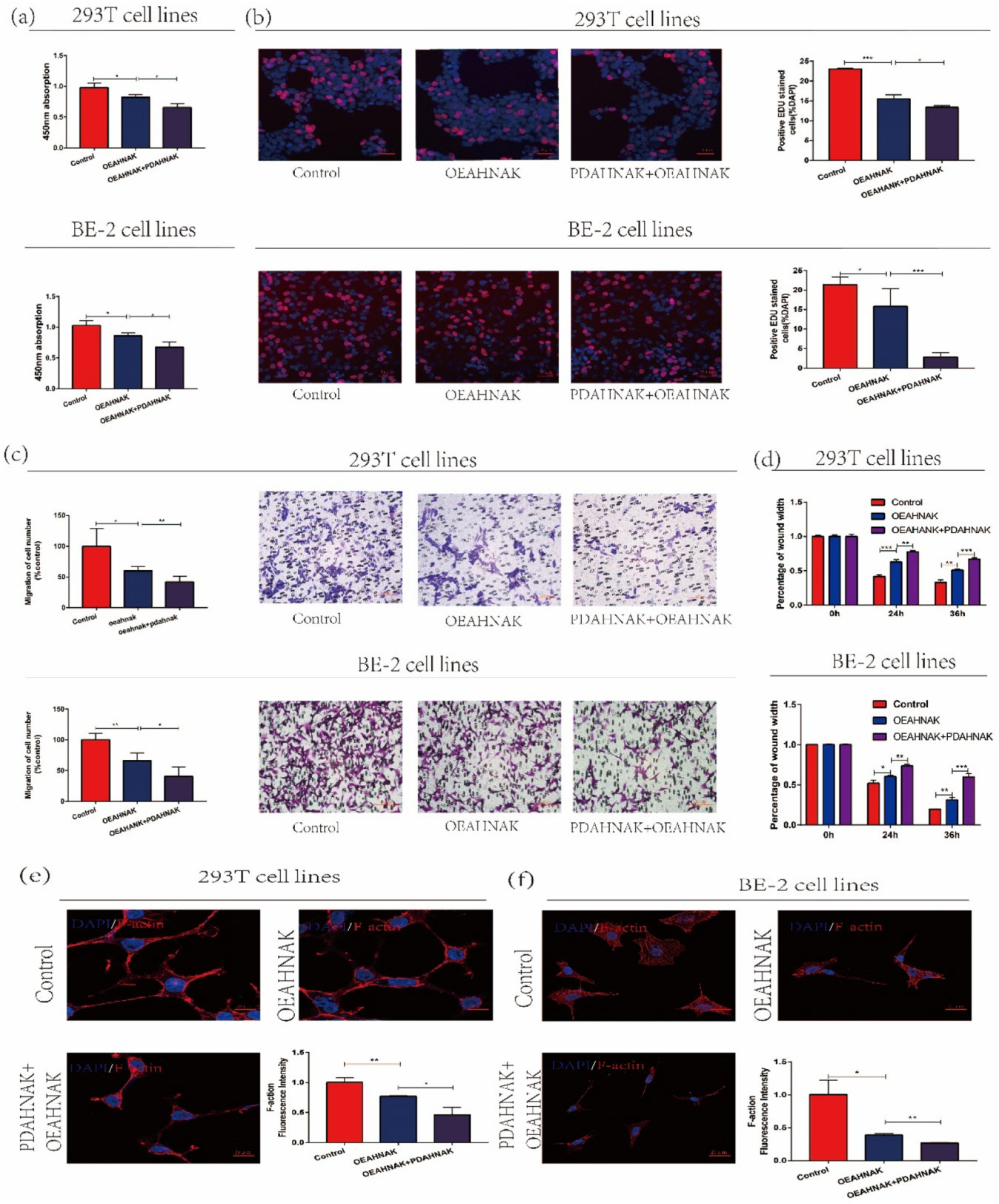


Figure 3. PDAHNAK enhanced the function of AHNAK. (a,b) CCK8 assay and EdU assay showed that PDAHNAK enhanced the inhibition function of AHNAK. Scale bar, 50 μm. (c) Co-transfection PDAHNAK enhanced the inhibition of AHNAK in cell migration. Scale bar, 100 μm. (d) Wound healing assay. (e,f) F-actin cytoskeleton was visualized with rhodamine phalloidin staining in human 293T and BE-2 cells. PDAHNAK enhanced the effect of AHNAK on the cytoskeleton. Scale bar, 20 μm.

°C for 30 min; then, wash three times with the washing buffer, and add 100 μL of the bottom to each well (incubate for 60 min in the dark at room temperature); finally, add the stopping solution. We measure the absorbance of the sample at 450 and

https://doi.org/10.1021/acs.jproteome.0c00811
J. Proteome Res. 2021, 20, 2308−2318

650 nm, where OD620 is the reference wavelength and OD450 is the detection wavelength. Finally, the difference between OD450 and OD620 was used as the relative concentration of each sample.

2.9. Western Blot Assay

The proteins were extracted with a RIPA buffer (Beyotime, Nantong, China). Then, we examined the protein concentration with a BCA kit (Beyotime). The proteins were electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separation and transferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore, Billerica, MA, USA). In the next step, the PVDF membranes were blocked in 5% skimmed milk for 60 min and incubated at 4 °C overnight with the primary antibodies [anti-GAPDH (1:1000, AG019-1, Beyotime), anti-ERK1/2(1:2000, #4696, Cell Signaling Technology), anti-p-ERK1/2 (1:2000, #4370, Cell Signaling Technology), and anti-AHNAK (1:1000, sc-390743, Santa Cruz Biotechnology)]. Later, the PVDF membranes were washed with Tris-buffered saline with Tween 20 (TBST) buffer (1× TBST) three times and then were incubated with secondary antibodies (Beyotime) for about 60 min at room temperature.

2.10. Immunofluorescence Staining

Using 4% paraformaldehyde, the control and transfected cells were fixed. After exposing cells to 0.5% Triton X-100 for 30 min, we incubated cells in rhodamine phalloidin (1:1000, Invitrogen) for 2 h. After washing three times, nuclei were stained with 4′,6-diamidino-2-phenylindole for 5 min. Then, the cells can be stored at 4 °C for 1 week, and pictures were captured with confocal laser scanning.

2.11. Polypeptide Injection and Observation of the Enteric Nerve

10 μM peptides were microinjected at the single-cell stage of zebrafish. At the same time, a wild-type control group and a ddH_2O injection group were set up. Then, we collected zebrafish embryos of 3, 4, and 5 dpf for observation. First, the fresh fish was washed three times with PBS containing 1% Triton for 5 min each. The fish was then quickly rinsed with ice water for 5 min and permeabilized with ice acetone for 10 min and placed at −20°. Next, we washed with water for 5 min and then with PBS containing 1% Triton for 5 min. After blocking the fish for 1.5 h at normal temperature (10% sheep serum +1% DMSO), the fish was incubated with anti-HUC/HUD at 4 °C for 4 days. Finally, the fish were incubated overnight with a secondary antibody and photographed with a stereomicroscope.

2.12. Statistical Analysis

ImageJ 1.8.0 and GraphPad Prism 7.0 were used for statistical analysis. All data are presented as the mean ± SD from three independent experiments. In all relevant experiments, statistical differences between data sets were assessed by the chi-square test or Student's *t*-test. *P*-value < 0.05 was considered statistically significant.

3. RESULTS

3.1. Overexpression of PDAHNAK in HSCR

The polypeptide sequence is "EGPEVDVNLPK" and aligned using SMART (http://smart.embl.de/#) (Figure 1a). Bioinformatics analysis of PDAHNAK by ProtParam revealed that PDAHNAK consists of 11 amino acids with a molecular weight of 1196.32 Da and an isoelectric point of 4.14 (Figure S2). The instability coefficient is 28.74. The liposoluble index is 88.18, and the hydrophilic average of hydropathicity (GRAVY) is −0.845 (Figure S2). The hydrophilicity of PDAHNAK is poor (Figure S2). The above results indicated that PDAHNAK is a highly stable and soluble endogenous peptide.

The patient's age and sex were obtained at the time of sample collection, and there was no statistical difference between HSCR patients and matched controls (Table 1). The PDAHNAK expression was measured in HSCR tissues (n = 24) and controls (n = 24) by ELISA. As shown in Figure 1b, PDAHNAK was overexpressed in HSCR-stricture tissues compared to HSCR-dilated and control tissues.

3.2. Effects of PDAHNAK on Cell Proliferation and Migration

The functional effects of PDAHNAK were detected in 293T and BE2 cell lines transfected with PDAHNAK *in vitro*. First, immunofluorescence staining confirmed that FITC-labeled PDAHNAK was able to enter into the cell through the membrane (Figure 1c). Then, 293T and BE-2 cells were exposed with different concentrations of PDAHNAK (0, 1, 10, 50, and 100 μg/ml), and the results showed that PDAHNAK can significantly inhibit cell migration and proliferation when the concentration of PDAHNAK reaches 10 μg/mL (Figure S3a,b). Therefore, overexpression of PDAHNAK in 293T and BE-2 cells was all performed at a concentration of 10 μg/mL in the study. As shown in Figure 2a,b, CCK8 and EdU experiments show that the proliferation ability of the cells exposed with PDAHNAK was significantly inhibited. Similarly, transwell and scratch experiments show that the cells exposed with PDAHNAK have significantly reduced migration ability (Figure 2c,d). Details of the wound healing assay are shown in Figure S4. Unlike the above results, the flow cytometry assay showed that there was no statistical difference in cell apoptosis and cycle rate between the cells treated with PDAHNAK and the control (Figure S5a,b). The cytoskeletal remodeling was characterized with rhodamine-labeled phalloidin. The cell cytoskeleton elements were attenuated after transfection with PDAHNAK (Figure 2e).

3.3. Synergism between PDAHNAK and AHNAK

To explore the relationship between AHNAK and PDAHNAK, 293T and BE-2 cells were first transfected with plasmids to overexpress AHNAK. We found that plasmids could upregulate the AHNAK expression (Figure S5c). Each experiment was divided into three groups including "control", "AHNAK", and "AHNAK and PDAHNAK". CCK8 and EdU assays showed that PDAHNAK could enhance the weakening effect of AHNAK on proliferation (Figure 3a,b). According to the results of transwell and wound healing assays (for details, see Figure S6), cell migration in the "AHNAK and PDAHNAK" group was significantly reduced compared to the group of "overexpression of AHNAK" (Figure 3c,d). The cytoskeletal remodeling was characterized with rhodamine-labeled phalloidin. As shown by the results, PDAHNAK could enhance the weakening effect of AHNAK on the cytoskeleton elements (Figure 3e,f).

3.4. PDAHNAK Inhibits the Phosphorylation of Protein ERK1/2

By the western blot, the expressions of the proteins ERK1/2 and p-ERK1/2 in the control group and PDAHNAK group were compared. The western blot showed that compared with the control group, there was no significant difference in the expression of cellular protein ERK1/2, while the expression of p-ERK1/2 was significantly reduced in the PDAHNAK group. There were significant statistical differences (Figure 4a). At the

https://doi.org/10.1021/acs.jproteome.0c00811
J. Proteome Res. 2021, 20, 2308−2318

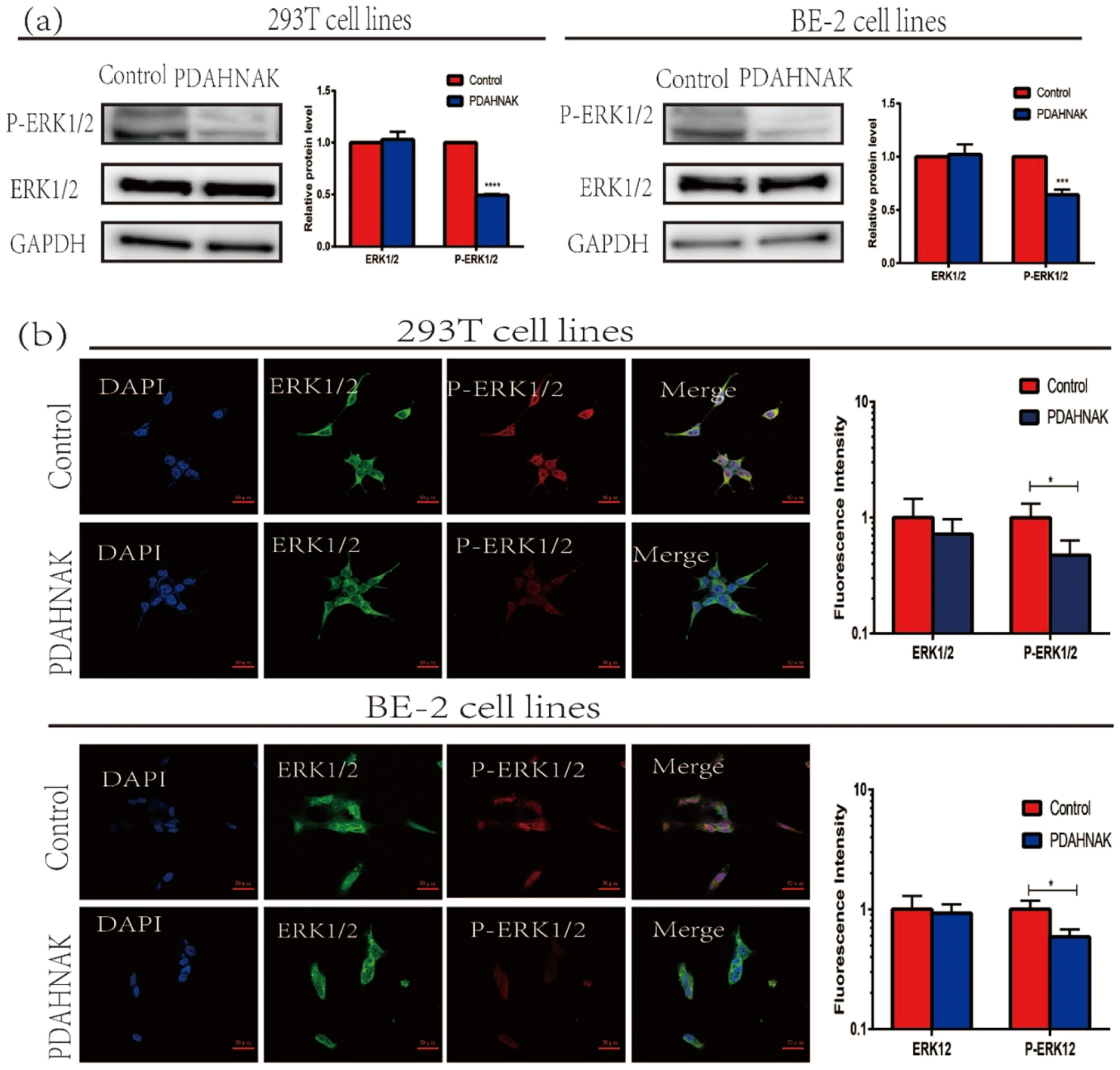


Figure 4. PDAHNAK inhibits phosphorylation of ERK1/2. (a) Relative levels of ERK1/2 were compared between the control and PDAHNAK-transfected 293T and BE-2 cells by western blotting. (b) Relative levels of ERK1/2 were compared between the control and PDAHNAK-transfected 293T and BE-2 cells by cellular immunofluorescence. Scale bar, 50 μm.

same time, the research team further verified through immunofluorescence experiments. The assay found that the relative fluorescence brightness of ERK1/2 in cells exposed PDAHNAK was not different from that in the control group, while the relative fluorescence brightness of p-ERK1/2 was significantly reduced (Figure 4b). Western blot and cellular immunofluorescence assays confirmed that PDAHNAK markedly suppressed the phosphorylation of ERK1/2 proteins in BE-2 and 293T cells.

3.5. PDAHNAK Inhibits Proliferation and Migration of Zebrafish Intestinal Nerve Cells

We selected fish at 3, 4, and 5 dpf after fertilization for observation. Our experimental results show that at 3 dpf, the intestinal nerve cells of the control group and the ddH_2O injection group have migrated to the cloaca, but the enteral nerve cells of the PDAHNAK group have not yet appeared in the intestine (Figure 5A). At 4 dpf, the enteral nerve cells of zebrafish injected with PDAHNAK can be seen, but the number is significantly less than that of the control group and the ddH2O group (Figure 5B). The experimental results observed at 5 dpf are the same as those observed at 4 dpf (Figure 5C). These results indicate that PDAHNAK can to some extent inhibit the proliferation and migration of the enteral nerve in zebrafish.

4. DISCUSSION

HSCR is a congenital gastrointestinal malformation with multiple genetic inheritance characteristics.[12] Its pathological feature is the lack of ganglion cells in the distal intestinal

https://doi.org/10.1021/acs.jproteome.0c00811
J. Proteome Res. 2021, 20, 2308−2318

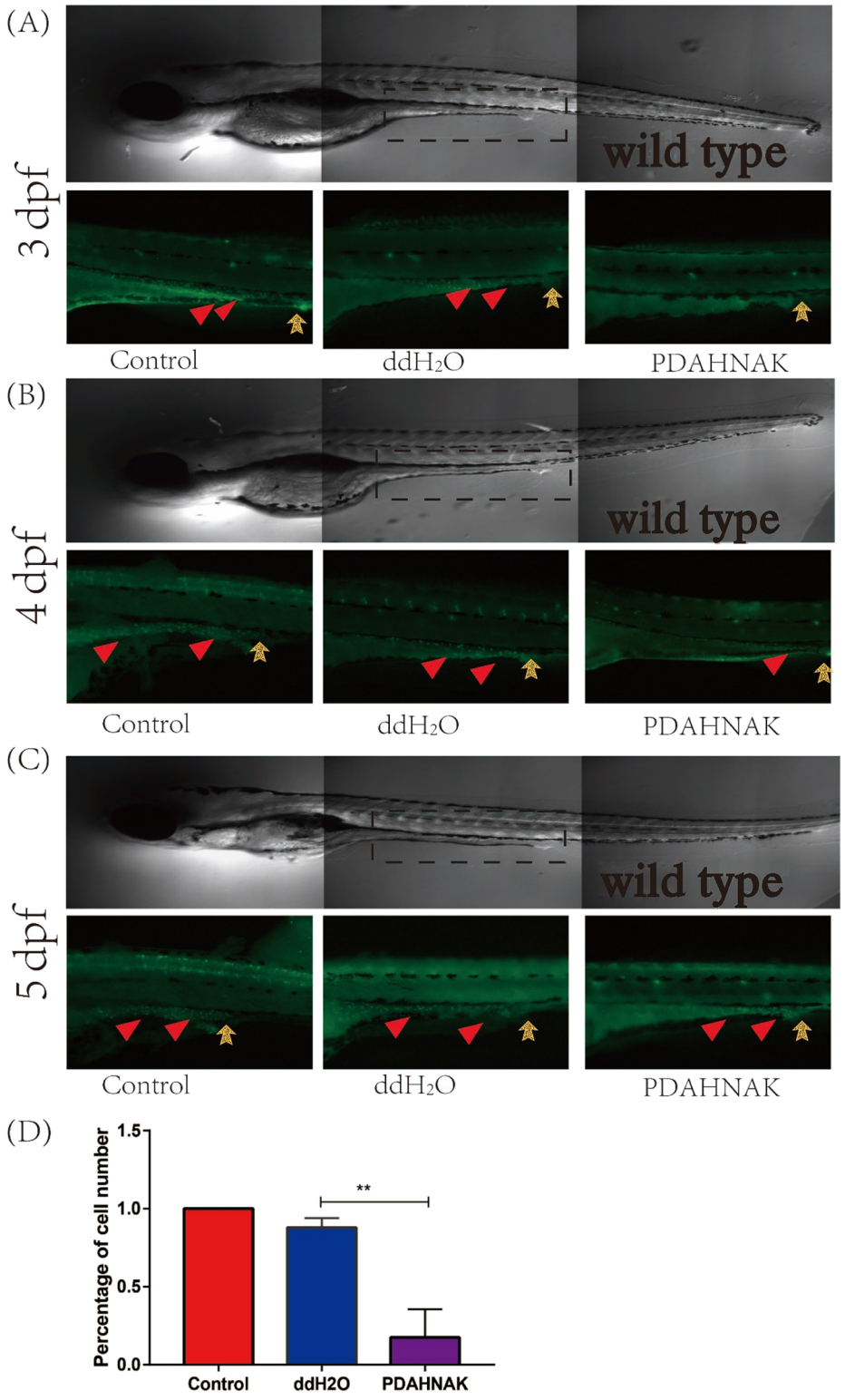


Figure 5. PDAHNAK inhibited the migration and proliferation of zebrafish enteric nerve cells. The yellow arrows indicate the location of the cloaca, and the red arrows indicate the intestinal neurons. 10 μM peptides were microinjected at the single-cell stage of zebrafish. (A) At 3 dpf, intestinal neurons have migrated to the cloaca in the wild-type and ddH_2O-injected groups, but intestinal neurons in the PDAHNAK-injected group have not been seen. (B) At 4 dpf, intestinal neurons in all three groups reached the cloaca position, but the number of neurons in the wild-type and ddH_2O-injected groups was significantly higher than that in the PDAHNAK-injected group. (C) At 5 dpf, the migration and proliferation of intestinal neurons are the same as in (B). (D) PDAHNAK inhibits the proliferation of ENCCs in zebrafish.

segment.[13,14] The absence of enteric neurons can cause the tonic contraction of the intestinal tract, which can lead to intestinal obstruction and other clinical manifestations.[15,16] The current treatment of HSCR is mainly surgical resection of the non-ganglionic intestinal tube, but postoperative incontinence and constipation and other complications are common.[17,18] The

https://doi.org/10.1021/acs.jproteome.0c00811
J. Proteome Res. 2021, 20, 2308−2318

genetic background of HSCR is very complex. Most HSCR cases are sporadic (80%), and the rest are familial.[19]

In the embryonic stage, neural crest cells move to different axial levels (cranial, cardiac, vagal, torso, and bone) and migrate throughout the embryo, thereby colonizing multiple organs and primitively differentiating into multiple cell types.[20] The ganglion cells of the enteric nervous system (ENS) mainly come from vagal crest cells. Around the 3rd week of human pregnancy, vagal crest cells proliferate and enter the foregut and finally complete colonization of the entire intestine around the 7th week.[17] The exact mechanism of vagal crest cell colonization is unclear. However, it is well known that the signal transduction pathways, especially the RET/GFRα1/GDNF[21] and EDNRB/ECE1/EDN3[22] signaling pathways, are crucial. At present, it is believed that gene mutations are the main cause of HSCR, but these genes and their mutations can only partly explain the pathogenesis of HSCR. There are still other pathogenic factors to be discovered.

With the development of peptidomics, the role of endogenous peptides with relatively simple structures in the occurrence and development of diseases has attracted much attention. Peptides have an irreplaceable value in exploring the mechanism of disease and developing new therapeutic drugs. Some researchers have discovered a peptide Humanin composed of 24 amino acid residues in the cDNA library of an Alzheimer disease (AD) patient, which can reverse the mutations caused by a variety of AD-related genes.[23] In recent years, many scholars have found that changes in the environment of the intestinal wall are closely related to the occurrence of HSCR. Studies have confirmed that extracellular matrix proteins play an important role in the early migration of ENCCs. For example, laminin plays an important role in the migration, proliferation, and differentiation of ENCCs.[24] In addition to these higher-molecular-weight proteins, biologically active peptides also exist in the microenvironment of the intestinal wall. They are derived from proteolysis and play an important role in life activities. However, whether these endogenous peptides affect the migration of ENCCs is related to the occurrence of HSCR.

In this study, we found that the peptide with the sequence "EGPEVDVNLPK" not only has significantly increased expression but also has more stable physical and chemical properties (Figure S1), so we chose it for further research. Because there is no unified naming convention, we temporarily refer to this endogenous peptide derived from AHNAK as PDAHNAK. Next, through ELISA experiments, we studied the upregulation of PDAHNAK expression in the HSCR stenosis intestinal tissue compared with normal controls and the HSCR dilatation intestinal tissue. In addition, FITC-labeled PDAHNAK was synthesized *in vitro*, and it was detected under a fluorescence microscope that PDAHNAK synthesized *in vitro* could enter cells through diffusion and endocytosis. In addition, the results of *in vitro* cell function experiments confirmed that the migration and proliferation functions of cells transfected with PDAHNAK were inhibited.

AHNAK, as a focal adhesion-related protein, can participate in the binding of cadherin and affect the activity of cadherin, thereby affecting the assembly of cytoskeletal elements. Studies have found that AHNAK regulates cell migration by affecting the stability of the cytoskeleton.[25] The endogenous polypeptide PDAHNAK is derived from its precursor protein AHNAK. To verify whether PDAHNAK affects the cytoskeleton, we have PDAHNAK transfected in the cell line to observe whether the cytoskeleton was affected. The experimental results show that after transfection with PDAHANK, the assembly of cytoskeletal elements is significantly reduced. In addition, studies have shown that AHANK can inhibit the proliferation and migration of cancer cells in various cancers (lung cancer,[26] breast cancer,[27] etc.). Combined with previous research of ours (unpublished), the expression of AHNAK in the stenosed segment of HSCR is higher than that of the normal intestinal segment, and after overexpression of AHNAK, the proliferation and migration of nerve-related cells are inhibited. This study further found that PDAHNAK and AHANK have a synergistic effect in inhibiting cell proliferation and migration and cytoskeletal activity through *in vitro* cell function tests and rhodamine-labeled phalloidin experiments. After transfection of PDAHNAK in cells, the inhibition of AHNAK on cell proliferation and migration and the cytoskeleton was enhanced.

Previous studies have shown that the ERK1/2 signaling pathway plays an important role in the proliferation and migration of enteric nerve cells, and the activity of ERK1/2 is closely related to the occurrence of HSCR. Rouleau et al. found that ERK1/2 phosphorylation of the intestinal tract tissue of congenital megacolon stenosis significantly decreased compared with the normal control colon tissue.[28] Another study found that ERK1/2 is involved in the development of the ENS, and the phosphorylation level of ERK1/2 is reduced, which can affect the proliferation and differentiation of enteric nerve precursor cells and lead to the occurrence of congenital megacolon.[29] In order to clarify the underlying mechanism of PDAHNAK's role, we verified that PDAHNAK can interfere with cell proliferation and migration via the ERK1/2 pathway. Western blot and immunofluorescence co-localization experiments showed that after overexpression of PDAHNAK in the cells, the amount of ERK1/2 total protein in the cells was not affected, but the phosphorylation level of ERK1/2 was reduced, indicating that PDAHNAK can inhibit ERK1/2 activity, and PDAHNAK can participate in the occurrence of congenital megacolon by affecting the phosphorylation of ERK1/2.

Zebrafish is currently a good biological model for the study of congenital diseases due to its high homology with human genes.[30,31] Both the zebrafish models can be used to screen and evaluate compounds with clear targets, and genetic verification can also be performed for complex diseases with unknown mechanisms.[32] In this study, we used zebrafish embryos to perform PDAHNAK in vivo functional experiments, injected 5 ng of PDAHNAK at the single-cell stage of zebrafish, and collected zebrafish embryos for observation on days 3, 4, and 5. The results indicate that PDAHNAK inhibits the proliferation of ENCCs and slows the migration speed of ENCCs in zebrafish.

In summary, we demonstrated for the first time that the endogenous polypeptide PDAHNAK is highly expressed in the stenosed segment of HSCR intestines, and overexpression of PDAHNAK can impair cell proliferation and migration. In addition, a synergistic relationship between PDAHNAK and AHNAK was discovered. PDAHNAK enhanced the inhibitory effect of AHNAK on cell proliferation and migration and the cytoskeleton. Furthermore, zebrafish models further verified that PDAHNK inhibits the migration and proliferation of ENCCs in vivo.

However, this is only one of the many endogenous peptides derived from the protein AHNAK. Whether other peptides have the same or opposite functions remains to be explored. At present, the specific pathogenesis of HSCR is still unclear, and patients still have more postoperative complications after surgical treatment. Researchers must face great challenges in

https://doi.org/10.1021/acs.jproteome.0c00811
J. Proteome Res. 2021, 20, 2308−2318

their field to find pharmacological and therapeutic methods for specific molecules or steps. From the perspective of peptides in the intestinal wall microenvironment, this study provides a new perspective for the mechanism research and treatment of HSCR. At the same time, from the perspective of peptides, it can further strengthen our understanding of its prerequisite proteins.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.0c00811.

Expression levels of AHNAK; schematic overview of choosing the polypeptide sequence "EGPEVDVNLPK" as the research object; CCK8 and Transwell assay showing effects of different concentrations of PDAHNAK on cell proliferation and migration; wound healing assay; BD Biosciences FACS Calibur Flow Cytometry performed to evaluate the difference of cell cycle and apoptosis rate between cells treated with PDAHNAK and the control; transfection efficiency of AHNAK evaluated by western blotting; and wound healing assay (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Hongxing Li − *Department of Pediatric Surgery, Children's Hospital of Nanjing Medical University, Nanjing 210008, China; State Key Laboratory of Reproductive Medicine, Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, China*; Phone: +86-25-83117231; Email: hx8817@126.com; Fax: +86-25-86868427

Weibing Tang − *Department of Pediatric Surgery, Children's Hospital of Nanjing Medical University, Nanjing 210008, China; State Key Laboratory of Reproductive Medicine, Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, China*; orcid.org/0000-0002-3582-3408; Phone: +86-25-83117354; Email: twbcn@njmu.edu.cn; Fax: +86-25-86868427

Authors

Yuhan Li − *Department of Pediatric Surgery, Children's Hospital of Nanjing Medical University, Nanjing 210008, China; Department of Pediatric Surgery, Xin Hua Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200240, China*

Xiurui Lv − *Department of Pediatric Surgery, Children's Hospital of Nanjing Medical University, Nanjing 210008, China; School of Medicine & Dentistry, University of Rochester, Rochester 14642, United States*

Huan Chen − *Department of Pediatric Surgery, Children's Hospital of Nanjing Medical University, Nanjing 210008, China; State Key Laboratory of Reproductive Medicine, Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, China*

Zhengke Zhi − *Department of Pediatric Surgery, Children's Hospital of Nanjing Medical University, Nanjing 210008, China; State Key Laboratory of Reproductive Medicine, Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, China*

Zhonghong Wei − *Department of Pediatric Surgery, Children's Hospital of Nanjing Medical University, Nanjing 210008, China*

Binyu Wang − *Department of Pediatric Surgery, Children's Hospital of Nanjing Medical University, Nanjing 210008, China; State Key Laboratory of Reproductive Medicine, Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, China*

LingLing Zhou − *Department of Pediatric Surgery, Children's Hospital of Nanjing Medical University, Nanjing 210008, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jproteome.0c00811

Author Contributions

Y.L., X.L., H.C., Z.Z., and Z.W.: investigation and visualization. B.W.: investigation. L.Z.: formal analysis and validation. Y.L., X.L., H.C.: conceptualization, methodology, and writing-original draft preparation. H.L., W.T.: conceptualization, supervision, and writing-reviewing and editing. All authors read and approved the final manuscript.

Funding

This work was supported by NSFC 82001590 (Zhengke Zhi), NSFC 81701493 (Hongxing Li), and NSFC 81870372 (Weibing Tang).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Xiaofeng Lv, Wei Li, and Hua Xie (Children's Hospital of Nanjing Medical University) for sample collection. This study was supported by the National Natural Science Foundation of China and the Key Research and Development (Social Development) Program of Jiangsu Province of China (BE2017609).

■ ABBREVIATIONS

HSCR, Hirschsprung's disease; ENCCs, enteric neural crest cells; "EGPEVDVNLPK", PDAHNAK, peptide derived from AHNAK; EdU, 5-ethynyl-20-deoxyuridine; ELISA, enzyme-linked immunosorbent assay

■ REFERENCES

(1) Chatterjee, S.; Kapoor, A.; Akiyama, J. A.; et al. Enhancer Variants Synergistically Drive Dysfunction of a Gene Regulatory Network In Hirschsprung Disease. *Cell* **2016**, *167*, 355−368.

(2) Fattahi, F.; Steinbeck, J. A.; Kriks, S.; et al. Deriving human ENS lineages for cell therapy and drug discovery in Hirschsprung disease. *Nature* **2016**, *531*, 105−109.

(3) Emison, E. S.; McCallion, A. S.; Kashuk, C. S.; et al. A common sex-dependent mutation in a RET enhancer underlies Hirschsprung disease risk. *Nature* **2005**, *434*, 857−863.

(4) Akbareian, S. E.; Nagy, N.; Steiger, C. E.; et al. Enteric neural crest-derived cells promote their migration by modifying their microenvironment through tenascin-C production. *Dev. Biol.* **2013**, *382*, 446−456.

(5) Fujiwara, N.; Nakazawa-Tanaka, N.; Miyahara, K.; Arikawa-Hirasawa, E.; Akazawa, C.; Yamataka, A. Altered expression of laminin alpha1 in aganglionic colon of endothelin receptor-B null mouse model of Hirschsprung's disease. *Pediatr. Surg. Int.* **2018**, *34*, 137−141.

(6) Breau, M. A.; Dahmani, A.; Broders-Bondon, F.; Thiery, J.-P.; Dufour, S. Beta1 integrins are required for the invasion of the caecum and proximal hindgut by enteric neural crest cells. *Development* **2009**, *136*, 2791−2801.

(7) Jin, J.; Bhatti, D. L.; Lee, K.-W.; et al. Ahnak scaffolds p11/Anxa2 complex and L-type voltage-gated calcium channel and modulates depressive behavior. *Mol. Psychiatry* **2020**, *25*, 1035−1049.

(8) Hohaus, A.; Person, V.; Behlke, J.; Schaper, J.; Morano, I.; Haase, H. The carboxyl-terminal region of ahnak provides a link between cardiac L-type Ca2+ channels and the actin-based cytoskeleton. *FASEB J.* **2002**, *16*, 1205−1216.
(9) Nonnenmacher, M.; Salmon, J.; Jacob, Y.; Orth, G.; Breitburd, F. Cottontail rabbit papillomavirus E8 protein is essential for wart formation and provides new insights into viral pathogenesis. *J. Virol.* **2006**, *80*, 4890−4900.
(10) Liu, Y.; Luo, B.; Shi, R.; et al. Nonerythropoietic Erythropoietin-Derived Peptide Suppresses Adipogenesis, Inflammation, Obesity and Insulin Resistance. *Sci. Rep.* **2015**, *5*, 15134.
(11) Matsumoto, A.; Pasut, A.; Matsumoto, M.; et al. mTORC1 and muscle regeneration are regulated by the LINC00961-encoded SPAR polypeptide. *Nature* **2017**, *541*, 228−232.
(12) Sribudiani, Y.; Chauhan, R. K.; Alves, M. M.; et al. Identification of Variants in RET and IHH Pathway Members in a Large Family With History of Hirschsprung Disease. *Gastroenterology* **2018**, *155*, 118−129.
(13) Gui, H.; Schriemer, D.; Cheng, W. W.; et al. Whole exome sequencing coupled with unbiased functional analysis reveals new Hirschsprung disease genes. *Genome Biol.* **2017**, *18*, 48.
(14) Heuckeroth, R. O. Hirschsprung's disease, Down syndrome, and missing heritability: too much collagen slows migration. *J. Clin. Invest.* **2015**, *125*, 4323−4326.
(15) Liu, J. A.-J.; Lai, F. P.-L.; Gui, H.-S.; et al. Identification of GLI Mutations in Patients With Hirschsprung Disease That Disrupt Enteric Nervous System Development in Mice. *Gastroenterology* **2015**, *149*, 1837−1848.
(16) Gershon, M. D. NPARM in PHOX2B: why some things just should not be expanded. *J. Clin. Invest.* **2012**, *122*, 3056−3058.
(17) Gershon, M. D. Developmental determinants of the independence and complexity of the enteric nervous system. *Trends Neurosci.* **2010**, *33*, 446−456.
(18) Griseri, P.; Patrone, G.; Puppo, F.; Romeo, G.; Ravazzolo, R.; Ceccherini, I. Rescue of human RET gene expression by sodium butyrate: a novel powerful tool for molecular studies in Hirschsprung disease. *Gut* **2003**, *52*, 1154−1158.
(19) Gath, R.; Goessling, A.; Keller, K. M.; et al. Analysis of the RET, GDNF, EDN3, and EDNRB genes in patients with intestinal neuronal dysplasia and Hirschsprung disease. *Gut* **2001**, *48*, 671−675.
(20) Amiel, J.; Lyonnet, S. Hirschsprung disease, associated syndromes, and genetics: a review. *J. Med. Genet.* **2001**, *38*, 729−739.
(21) Ruiz-Ferrer, M.; Torroglosa, A.; Núñez-Torres, R.; de Agustín, J. C.; Antiñolo, G.; Borrego, S. Expression of PROKR1 and PROKR2 in human enteric neural precursor cells and identification of sequence variants suggest a role in HSCR. *PLoS One* **2011**, *6*, No. e23475.
(22) Hofstra, R. M. W.; Wu, Y.; Stulp, R. P.; et al. RET and GDNF gene scanning in Hirschsprung patients using two dual denaturing gel systems. *Hum. Mutat.* **2000**, *15*, 418−429.
(23) Levine, Z. A.; Teranishi, K.; Okada, A. K.; Langen, R.; Shea, J.-E. The Mitochondrial Peptide Humanin Targets but Does Not Denature Amyloid Oligomers in Type II Diabetes. *J. Am. Chem. Soc.* **2019**, *141*, 14168−14179.
(24) Druckenbrod, N. R.; Epstein, M. L. Age-dependent changes in the gut environment restrict the invasion of the hindgut by enteric neural progenitors. *Development* **2009**, *136*, 3195−3203.
(25) Matza, D.; Badou, A.; Kobayashi, K. S.; et al. A scaffold protein, AHNAK1, is required for calcium signaling during T cell activation. *Immunity* **2008**, *28*, 64−74.
(26) Gu, J.; Mao, W.; Ren, W.; et al. Ubiquitin-protein ligase E3C maintains non-small-cell lung cancer stemness by targeting AHNAK-p53 complex. *Cancer Lett.* **2019**, *443*, 125−134.
(27) Davis, T.; van Niekerk, G.; Peres, J.; Prince, S.; Loos, B.; Engelbrecht, A.-M. Doxorubicin resistance in breast cancer: A novel role for the human protein AHNAK. *Biochem. Pharmacol.* **2018**, *148*, 174−183.
(28) Rouleau, C.; Matécki, S.; Kalfa, N.; Costes, V.; de Santa Barbara, P. Activation of MAP kinase (ERK1/2) in human neonatal colonic enteric nervous system. *Neurogastroenterol. Motil.* **2009**, *21*, 207−214.
(29) Tian, J.; Zeng, C.; Tian, Z.; et al. Downregulation of Protein Tyrosine Phosphatase Receptor Type R Accounts for the Progression of Hirschsprung Disease. *Front. Mol. Neurosci.* **2019**, *12*, 92.
(30) Cahill, T. J.; Choudhury, R. P.; Riley, P. R. Heart regeneration and repair after myocardial infarction: translational opportunities for novel therapeutics. *Nat. Rev. Drug Discovery* **2017**, *16*, 699−717.
(31) Demarest, S. T.; Brooks-Kayal, A. From molecules to medicines: the dawn of targeted therapies for genetic epilepsies. *Nat. Rev. Neurol.* **2018**, *14*, 735−745.
(32) MacRae, C. A.; Peterson, R. T. Zebrafish as tools for drug discovery. *Nat. Rev. Drug Discovery* **2015**, *14*, 721−731.

NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on April 15, 2021. Author's contact information and funding statement were corrected after ASAP, and the corrected version was reposted on April 15, 2021.

https://doi.org/10.1021/acs.jproteome.0c00811
J. Proteome Res. 2021, 20, 2308−2318