The Innovation, Volume *5*

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MATERIALS AND METHODS

Cell lines and cell culture

Malignant pancreatic cell lines (PANC-1 and Mia PaCa-2) and nonmalignant human pancreas cell line (HPNE) were obtained from the American Type Culture Collection (Manassas, Virginia). The cells were cultured in DMEM medium (Hyclone, GE Healthcare Life Sciences) with HPNE cells having 0.1 ng/ml EGF (Novus Biologicals, USA) included. All cultures were supplemented with 10% fetal bovine serum (FBS; Life technology, Thermo Fisher Scientific Inc.), penicillin (1 U), and streptomycin (1 μg/ml). All cells were maintained in a humidified incubator with 5% CO_2 at 37ºC. All cell lines were cultured in triplicate under the same conditions and then harvested to collect independent exosome samples.

Cell viability assay

Cells were seeded in 96-well plates at a density of 10^4 cells/well. After 24 hr, culture medium with increasing concentrations of treatment was added to the treatment group for 48 hr. Cell viability was accessed by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instructions. Briefly, a mixture of 10 μl of CCK-8 and 190 μl media was added into each well and the cells were incubated for another 1 hr. The absorbance of each well was measured at 450 nm using a microplate reader. Each experiment was performed in 6 repeats.

Flow cytometry for mitochondrial mass quantification

Cells were stained by Mitoview Green (Biotium, Inc.). Briefly, the cells in the culture dish were treated with 2 ml of Trypsin-EDTA solution for 3 min at 37 °C, and 2 ml growth medium was added after. The cell suspensions were centrifuged to obtain the cell pellet and washed three times with PBS before being incubated for 20 min at 37 °C in 200 nM dye in the medium. Cells were then subjected to flow cytometric analysis using a BD Accuri™ C6 (BD Bioscience). At least 10,000 cells were acquired from each sample. FITC channel was used to capture the signal from the green dye. Flowing Software (Turku Centre for Biotechnology) was used for the analysis of the cytometric data. The intensity was normalized to the mean of the control.

Proteomics analysis

The quantitative proteomics study of the treated and untreated cells were analyzed at Tulane Proteomics Core. Briefly, four replicates each for cell lysates were diluted to 1 μg/μl with 100 mM NH_4HCO_3 supplemented with 10 mM dithiothreitol, incubated at 37 °C for 1 hr, then mixed with 30 mM iodoacetamide, incubated in the dark for 30 min at room temperature before overnight digestion with 1 μg trypsin at 37 °C. Digestions were terminated by the addition of 0.1% trifluoroacetic acid and diluted to 0.25 μg/μl protein with H_2O/acetonitrile (95:5), centrifuged at 21,000 g for 20 min. The digested peptides were cleaned up using StageTips. The dried peptides were reconstituted with H_2O/acetonitrile (95:5) before LC-MS/MS analyses that employed about 500 ng peptide/injection. Samples were analyzed using Q Exactive HF-X Quadrupole-Orbitrap MS System (Thermo Fisher Scientific). The peptides were eluted with a linear gradient from 2.5 to 35% buffer B (80% ACN in 0.1% FA) over 45 min. Following the linear separation, the system was ramped up to 75% in the next 10 min followed by 100% in 2 min. Then it was re-equilibrated to 2.5% in 7 min. The MS1 scans were collected from 300–1650 m/z with an AGC target of (3E6), a resolution of 60, 000 at 200 m/z, and followed by a top-15 MS2 loop. MS/MS scans were collected with a resolution of 15, 000 at 200 m/z, with an AGC target of 100 000 (1E5), and a maximum injection time of 118 ms. The dynamic exclusion time was set for 30 seconds. All LC-MS/MS data were analyzed by label-free quantitation mode and searched using the Sequest HT algorithm within Proteome Discoverer 2.4 (Thermo Fisher Scientific) against *Homo sapiens* proteome database (UP000005640) to obtain peptide and

protein identifications using a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da. For all searches, trypsin was specified as the enzyme for protein cleavage, allowing up to two missed cleavages. Oxidation (M) and carbamidomethylation (C) were set as dynamic and fixed modifications, respectively. The peptide spectrum match and protein false discovery rate (FDR) was set to 0.01 and determined using a percolator node. Relative protein quantification of the proteins was performed using the Minora feature detector node with default settings using peptide spectrum matches (PSM). The intensity ratio and adjusted p-values were calculated and provided in the supplementary file 2. Also, the proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[1] partner repository with the dataset identifier PXD047958. The proteomics data used for uncovering the unbalanced distribution of the amino acids was adopted from our previous research.[2,3]

Determination of the therapeutic index of each amino acid

The therapeutic index (TI) of each amino acid (i) is defined as $TI_i = \frac{\sum_1^n (1-v_i^{Tumor})}{n} - \left(1 - v_i^{Nontumor}\right)$, where $v_i^{Nontumor}$ and v_i^{Tumor} are the maximum cell viability when treating the nontumor tissue cells (HPNE) and tumor cells, respectively; n is the number of PDAC cell lines tested.[4]

Cell viability/apoptosis/necrosis assay

20-30% confluent cells were treated with branch chain amino acids, histidine, and isoleucine and incubated at recommended humidity (5%) and temperature (37 °C) for 48 hr. We had four separate groups of treatments (1) No treatment, (2) Histidine only, (3) Isoleucine only (4) Histidine + Isoleucine. After 48 hr, we collected adherent and non-adherent cells and counted them using a hemocytometer to have 5×10^5 cells per tube. We used Abcam Apoptosis/Necrosis Assay kit (Catalog: ab176749, Abcam). Cells were centrifuged at 500×g for 5 min at 4 °C . The supernatant was discarded, and the cell pellet was resuspended in 200 µl assay buffer. For detecting viable cells, we added 1 µl Cytocalcein Violet 450 in each tube. For apoptosis and necrosis, 2 µl of Apopoxin Green and 1 µl of 7-AAD were added, respectively. Next, cells were incubated for 60 min at room temperature. An additional 300 µl assay buffer was added to each tube, and samples were analyzed in a BDFACS Melody flow cytometer. Results were analyzed in FlowJo analysis software (Version: 5.2), and changes are expressed as median fluorescent intensity.

Immunoblotting

Cells with 20-30% confluency were treated with branch chain amino acid for 48 hr at 5% humidity and 37 °C . After treatment, the media was discarded, and PBS was used to wash the cells twice before adding 100 µl of 2× cell lysis buffer (Catalog: 9803S, Bio-rad). Cells were scrapped, collected, and mixed in a 360° rotator for 30 min at 4 °C. Cell lysates were centrifuged at 15000 rpm for 15 min at 4 °C . The supernatant was collected, and the protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay Kit (Catalog: 23225, Thermo Fisher Scientific). 30 µg of total protein from each cell lysate was mixed with an equal volume of 2× SDS Lammeli sample buffer (Catalog: 1610737, Bio-rad) containing β-Mercaptoethanol and boiled at 100 °C for 5 min. Then the sample was loaded into 4-20% precast polyacrylamide gel (Catalog: 4561093; Bio-Rad) for electrophoresis. After separating, the protein samples were transferred to the PVDF membrane (Catalog: 88520; Thermo Fisher Scientific) using the Trans-Blot Turbo transfer system. Then, the membrane was blocked using 5% non-fat milk (Catalog: SC-2325; Santa Cruz Biotechnology) in TBST for 2 hr. The membrane was washed 3 times in TBST solution and incubated with primary antibody, XRN1 (Dilution: 1:2500, Cat: ab70259, Abcam) overnight at 4 °C . The next day, the membrane was washed and treated with an HRP-tagged secondary antibody for 2 hr at room temperature. After washing, the protein was visualized using ECL-2 (Catalog: PI80196) in the western blotting detection system. ImageJ was used to quantify the expression level of proteins. To dilute both

primary and secondary antibodies, 5% BSA (Catalog: BP1600; Fisher Scientific) in TBST was used. The dilution ratio was optimized before performing the experiments.

shRNA transfection

PANC-1, XRN1 knockdown cells were generated using shRNA plasmid (catalog: TF300419C, OriGene). 40-50% confluent cells in 6-well plates were transfected using TurboFectin 8.0 (Catalog: TF81001, OriGene). A complex solution of Turbofectin and shRNA plasmid was prepared before transfection. For each well in 6-well plates, 250 µl of DMEM, 2 µg of shRNA plasmid DNA, and 3 µl of Turbofectin 8.0 was mixed and incubated at room temperature for 15 min. After that complex solution was added to the well and incubated for 24 hr at 37 °C . 24 hr post transcription, cells were moved into the serum-containing medium with 1 µg/ml puromycin for selection. Once all the non-transfected cells were dead, cells were moved into a cell culture dish and grown in 1 µg/ml puromycin-containing medium. The individual colony was picked up using cloning cylinders. Immunoblotting was performed to confirm the percent knockdown of XRN1 expression in clones. GAPDH expression is used as the loading control. Control shRNA provided in the kit was used to transfect control cells.

pH measurement

Amino acids were dissolved in the cell culture medium with a concentration of 5mg/ml and were measured by calibrated pH meter.

Imaging for mitochondrial density quantification

Cells were incubated for 20 min at 37 °C in 50 nM MitoTracker Green FM (MTK, Life Technologies). High-power overlay imagery (DIC, Green, and Blue channels) with maximum Z-stacks projections were imaged under Zeiss Axio Observer Z1 LSM 700 using the same staining procedure.

Wound-healing and invasion assays

Confluent cells were scratch-wounded using a 20 µl pipette tip, and cell debris was removed by washing with PBS. Phase-contrast time-lapse images were taken every 30 min for 24 hr after scratching at specific wound sites using a microscope with a 10 × objective. The wound healing closure percentage was calculated by an ImageJ macro (Wound Healing Tool)[5] comparing the area at the start and end time points. Values were averaged from three separate readings at each time. A migration assay was performed in 12-well plates using a Quantitative Cell Migration™ Assay Kit (Chemicon International, USA) with an 8.0 µm pore size collagen-coated chamber membrane. The cells were seeded (1×10^5 cells in 0.3 ml of serum-free medium) in the upper chamber and cultured for 24 hr for attachment. The medium was then replaced by a fresh serum-free medium for another 24 hr in the lower chamber. The cells were incubated for 12 hr, and the number of cells that passed through the membrane was counted according to the manufacturer's instructions. An invasion assay was performed in 24-well plates using a BD Biocoat™ Matrigel™ Invasion Chamber (BD Biosciences, USA) with an 8.0 µm pore size polyethylene terephthalate (PET) membrane coated with Matrigel. The inserts were rehydrated by adding 0.5 ml of culture medium at 37 °C for 2 hr. The cells were seeded (5×10^5 cells in 0.5 ml of serum-free medium) in the upper chamber and cultured as described in the method for the migration assay. The number of seeded cells, culture conditions, and other items were also like those for the migration assay. The cells were incubated for 24 hr and the number of cells that passed through the membrane was counted according to the manufacturer's instructions. All experiments were performed in triplicate and independently. Cell images were analyzed using CellProfiler (http://www.cellprofiler.org) as in Figure S4. Briefly, cell images were converted to grayscale and subjected to noise reduction techniques to enhance edges and set appropriate thresholds for reducing noise before measuring the mean intensity per cell.

Animal experiments

The xenograft model was established with six to eight-week-old male nude mice (J:NU-Foxn1nu) purchased from The Jackson Laboratories (Bar Harbor, ME). Five mice were housed per cage in static disposable cages from Innovive (San Diego, CA). The mice's food and water were checked daily, and housing was changed weekly. The pancreatic cancer tumor was injected subcutaneously into the left flank with 4×10^6 PANC-1 cells suspended in 100 µl of serum-free media with 50% Matrigel to establish a subcutaneous pancreatic tumor. Mice were observed three times daily for the first seven days and tumor xenograft was confirmed by the presence of the tumor. After confirmation, mice were divided into four groups randomly: three treatment groups [AA, AA+GEM, GEM] and one control group [PBS]. Tumor volume and mice weight were measured every fifth day. Tumor volume was established by measuring length and width; the volume formula used was the modified ellipsoid volume formula (½ length × width^2). For AA and control groups, treatment was given by oral gavage. AA treatment was made from a combination of histidine (44 mg/ml) and isoleucine (22 mg/ml) dissolved in PBS. For the two groups with AA, oral gavage treatment was administered daily with 200 µl AA treatment using a 20 ga polypropylene feeding tube attached to a syringe. For the control group, oral gavage treatment was administered using the same method except with 200 µl PBS. For the GEM and AA+GEM groups, GEM was given by intraperitoneal injection. GEM groups were injected twice a week with 200 µl of GEM (15 mg/ml) dissolved in PBS. All treatments were administered for sixty days. Two days after the last treatment dose, the mice were sacrificed using an isoflurane overdose and cervical dislocation. Tumors were carefully excised using scissors and a scalpel and placed on a blank sheet to be photographed.

Enrichment analysis and pathway identification

The Reactome Knowledgebase (https://reactome.org/) entails molecular and pathological details of processes in various diseases. Reactome was used to evaluate the enrichment analysis and determine the pathway affected by the treatment following the documentation procedure on the reference website.[6]

Histological analysis

Tumor sections were stained with hematoxylin and eosin (H&E) for image analysis. The stained tumor tissue sections on the slides were analyzed using QuPath version 0.4.3, open-source software for digital pathology.[7] Briefly, the tumor image analysis was carried out by creating a workflow to detect necrotic cells based on H&E staining reflected on the tissue slide. The slide image was optimized for staining. Annotation and objects that distinguish necrotic tumor cells were created as a consequence of the staining and annotated accordingly. A training classifier was then used to model the workflow before applying the detection to the whole tissue section.

Statistics

Data analysis comparisons between the two groups were performed using an unpaired two-sample t-test. All data analysis was performed using Origin Pro software. Data are presented either as representative examples or means ± SEM of 3+ experiments. All comparison groups had equivalent variances. $p < 0.05$ was viewed to be statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

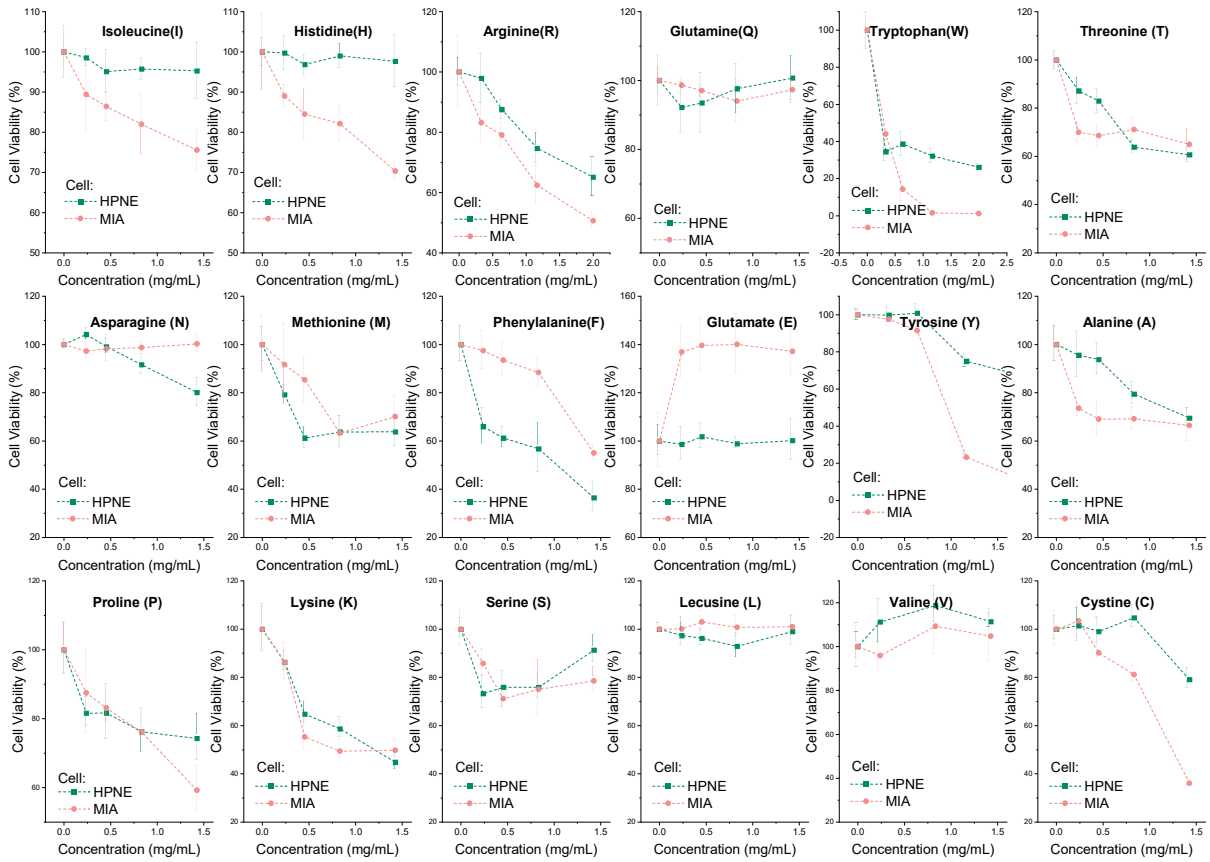

Figure S1. Cell viability is dose dependent of amino acids treatment. n=5. Isoleucine (I) and histidine (H) exhibited the highest selective toxicity toward cancer cells while being non-toxic to non-tumor cells.

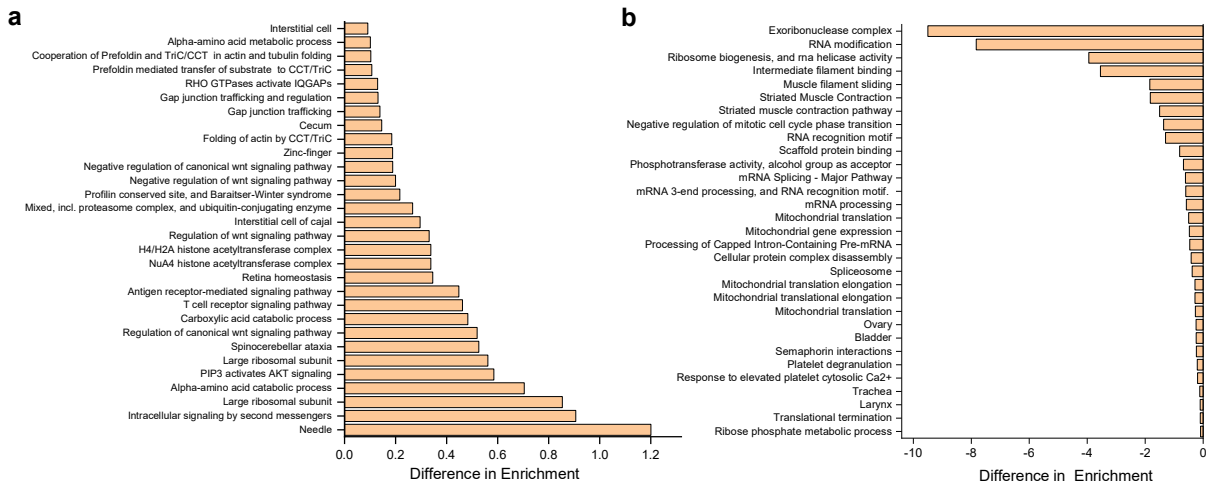

Figure S2. **Enrichment difference before and after the AA treatment. a**) highest enrichment. **b**) lowest enrichment.

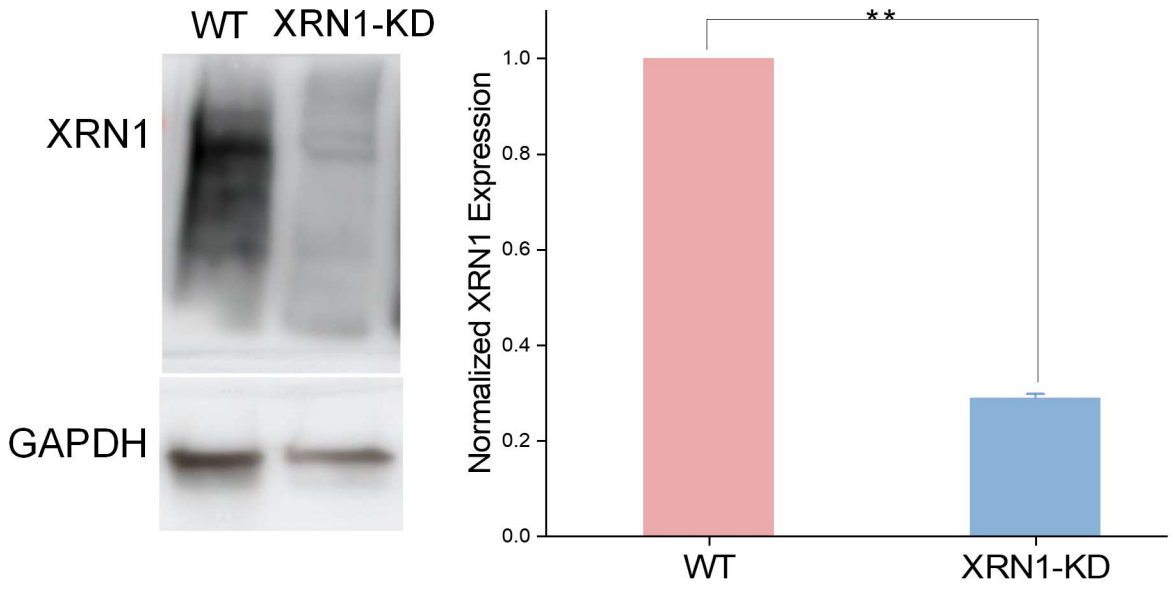

Figure S3. Western blot evaluating protein expression in XRN1 knockdown cells. WT: Wild type; XRN1-KD: XRN1 knockdown cells. $^{**}p < 0.01$.

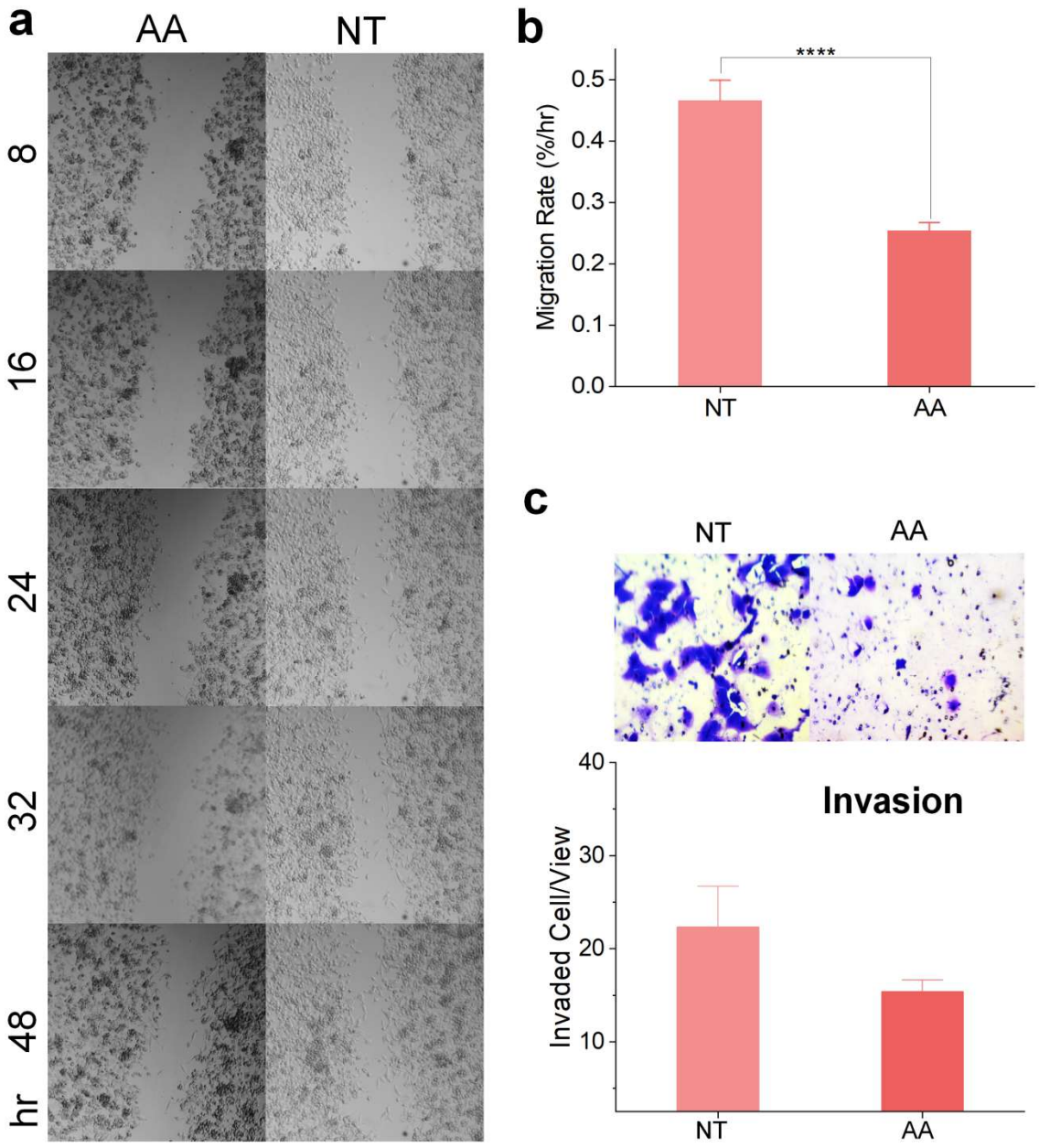

Figure S4. **AA treatment inhibits cell migration and invasion. a, b)** effect of AA treatment inhibiting cell migration. **c)** effect of AA treatment inhibiting invasion. AA – with amino acids treatment; NT – without amino acids treatment; ****$p < 0.0001$.

REFERENCES

1. Perez-Riverol, Y., Bai, J., Bandla, C., et al. (2022). The PRIDE database resources in 2022: A hub for mass spectrometry-based proteomics evidences. Nucleic Acids Res **50**(D1): D543-D552. DOI: 10.1093/nar/gkab1038.

2. Sun, D., Zhao, Z., Spiegel, S., et al. (2021). Dye-free spectrophotometric measurement of nucleic acid-to-protein ratio for cell-selective extracellular vesicle discrimination. Biosens Bioelectron **179**: 113058. DOI: 10.1016/j.bios.2021.113058.

3. Rasuleva, K., Elamurugan, S., Bauer, A., et al. (2021). β-Sheet Richness of the Circulating Tumor-Derived Extracellular Vesicles for Noninvasive Pancreatic Cancer Screening. ACS Sens **6**(12): 4489-4498. DOI: 10.1021/acssensors.1c02022.

4. Kashif, M., Andersson, C., Hassan, S., et al. (2015). In vitro discovery of promising anti-cancer drug combinations using iterative maximisation of a therapeutic index. Sci Rep **5**: 1-13. DOI: 10.1038/srep14118.

5. ImageJ. MRI Wound Healing Tool. http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool.

6. Gillespie, M., Jassal, B., Stephan, R., et al. (2022). The reactome pathway knowledgebase 2022. Nucleic Acids Res **50**(D1): D687-D692. DOI: 10.1093/nar/gkab1028.

7. Bankhead, P., Loughrey, M.B., Fernández, J.A., et al. (2017). QuPath: Open source software for digital pathology image analysis. Sci Rep **7**(1): 16878. DOI: 10.1038/s41598-017-17204-5.