Material and Methods

Cell culture and inhibitors

Human EC cell lines, including Ishikawa and Hec-1-B cells, were obtained from the Procell Life Science&Technology Co.,Ltd (Wuhan, China) and Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cells were maintained in complete RPMI-1640 medium containing 15% fetal bovine serum (FBS; Gibco, USA), 10 U/ml penicillin and 10 μg/ml streptomycin at 37 °C with 5% CO_2. According to the experimental requirements, Ishikawa and Hec-1-B cells were cultured in RPMI-1640 medium without glucose (Gibco, Thermo Fisher Scientific, MA, USA) and supplemented with 0 mM, 1 mM, 5 mM and 25 mM glucose (Sigma–Aldrich, St. Louis, MO, USA). The inhibitors involved in this study are listed below: vx-765, pyroptosis inhibitor, (S2228, Selleckchem, Houston, TX, USA); TEMPOL, mitochondrial ROS inhibitor, (ALX430-150, Enzo Life Sciences, Inc, USA); Oligomycin A, ATP synthase inhibitor, (10300-100, Agilent, CA, USA) and 2-Deoxy-D-glucose (2-DG), glycolysis inhibitor, (10300-100, Agilent, CA, USA).

RNA sequencing analysis

RNA sequencing was performed by Considerin (Wuhan, China). Total RNA was isolated from 17 EC tissues using TRIzol (Invitrogen, USA), and then RNA purity and integrity were evaluated. Subsequently, the

NEBNext® Ultra™ RNA Library Prep Kit (NEB, USA) was used to construct the RNA-seq library, and the purified library products were sequenced using the Illumina platform. Differentially expressed genes between EC patients with normal glucose (NG) and EC patients with diabetes mellitus (DM) were calculated using DESeq2 software (v 3.3) [log2 (fold change) > 0, $P<0.05$]. Furthermore, 2742 upregulated genes and 1830 downregulated genes were chosen for further analysis.

Cell Counting Kit-8 (CCK-8) assay

CCK-8 (Beyotime Biotechnology, Shanghai, China) assays were performed to evaluate cell proliferation. Briefly, 5000 cells were seeded in each well of a 96-well plate and then grown to approximately 80% confluence. After an incubation for 24 h at 37 °C, 10 μL of CCK-8 reagent were added to each well and incubated for 2 h at 37 °C in the dark. The optical density (OD) value was measured at a wavelength of 450 nm using a spectrophotometer.

Mito-SOX assay

MitoSOX Red (Thermo Fisher, M36008, Massachusetts, USA) was used to measure mitochondrial ROS production. Cells were seeded and then grow to approximately 80% confluence. After treatment with different agents according to the different experimental groups, MitoSOX Red reagent (10 μM) was added to cell and incubated for 20 min at 37 °C in the dark. Mitochondrial ROS accumulation in cells was imaged after three

washes with 1x PBS.

Hoechst 33342/PI fluorescent staining

Hoechst 33342/PI double fluorescent staining (C1056, Beyotime Biotechnology, Shanghai, China) was performed to measure pyroptosis. After treatment with different agents according to the different experimental groups, the cells were stained with 6 μL of Hoechst 33342 solution and 6 μL of PI (propidium iodide) at 4 °C in the dark for 20 min. Images were captured with a Leica DMI4000 microscope.

Lactate dehydrogenase (LDH) release assay

LDH Release Assay Kit (C0016, Beyotime Biotechnology, Shanghai, China) was used according to the manufacturer's instructions. After an incubation at 37 °C for 24 h, 200 μL of cell culture medium containing LDH releasing agent were incubated with the cells for 30 min, and then the absorbance was measured at 490 nm on a spectrophotometer.

Seahorse assay

The extracellular acidification rate (ECAR) of cells was measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA) according to the manufacturer's instructions. Briefly, EC cells were plated in XF24 cell culture plates (Agilent) at a density of 1×10^4 cells/well. Then, the cells were treated with different agents according to the different experimental groups. After Seahorse XF assay medium was added to each well and incubated for 1 h at 37 °C, ECAR was

detected following sequential additions of glucose (10 mM), oligomycin (1 μM) and 2-DG (50 mM).

Dual luciferase Reporter Assay

For the assessment of miR-876-5p binding to the HKDC1 3′-UTR or HOXC-AS2, the potential miR-876-5p binding sites in the HKDC1 3′-UTR or HOXC-AS2 were mutated and cloned into luciferase expression plasmids (GenePharma, Shanghai, China). Cells were cotransfected with the vectors or hsa-miR-876-5p mimics using Lipofectamine 2000 for 48 h. Luciferase activities were measured using a luminometer (Lumat LB9507) with a Dual-Luciferase Reporter Assay kit (RG027, Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions.

Fluorescent in situ hybridization (FISH)

According to the FISH Kit (GenePharma, Shanghai, China) manufacturer's instructions, cells were cultured on coverslips, washed three times with 1x PBS, fixed with 4% paraformaldehyde, and permeabilized. After blocking with prehybridization solution at 37 °C for 30 min, the cells were incubated with hybridization solution containing HOXC-AS2 probes overnight and washed with 1x PBS for three times, and DAPI (1:200, C1002, Beyotime, Shanghai, China) was added to stain the nuclei at 37 °C for 10 min.

RNA immunoprecipitation (RIP)

RIP assays were performed using an RNA immunoprecipitation kit (Bes5101, BersinBio, Guangzhou, China) according to the manufacturer's instructions. Briefly, 2×10^7 cells were collected and lysed, after removing DNA, an anti-AGO2 antibody (Abcam, ab186733, 1:50) and control IgG were used for coprecipitation overnight at 4 °C, and coprecipitated miR-876-5p, miR-3167 and HOXC-AS2 were measured using qRT-PCR.

Transwell migration assays

For migration assays, cells suspended in DMEM were plated in the upper wells of modified Boyden chambers, and the lower chamber contained the experimental reagents in 5% FBS+DMEM. After 24 h of culture, the cells were fixed with 4% paraformaldehyde and stained with a 0.4% crystal violet solution. The nonmigrated cells on the upper part of the filter were removed, and the number of stained, migrated cells was counted under an inverted microscope.

Small interfering RNA (siRNA) design and transfection

Cells were transfected with siRNAs and Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. SiRNAs were designed and synthesized by GenePharma (Suzhou, China). The target sequences were as follows:

Negative control (NC) siRNA, sense: 5′-UUCUCCGAACGUGUCACGUTT-3′ and antisense:

5′-ACGUGACACGUUCGGAGAATT-3′;

HOXC-AS2 siRNA1, sense: 5′-GGUGGCAGACCAGGGCUUUTT-3′ and antisense: 5′-AAAGCCCUGGUCUGCCACCTT-3′;

HOXC-AS2 siRNA2, sense: 5′-GGAAGCGGAGGUGGCGAAATT-3′ and antisense: 5′-UUUCGCCACCUCCGCUUCCTT-3′;

HOXC-AS2 siRNA3, sense: 5′-CCAAUGAAUGAAAGUGCAATT-3′ and antisense: 5′-UUGCACUUUCAUUCAUUGGTT-3′;

HOXC-AS2 siRNA4, sense: 5′-CCUGCAGAUUUCCCUCAAATT-3′ and antisense: 5′-UUUGAGGGAAAUCUGCAGGTT-3′;

HOXC-AS2 siRNA5, sense: 5′-GCAGAAACUGAGUUCUCUUTT-3′ and antisense: 5′-AAGAGAACUCAGUUUCUGCTT-3′;

HOXC-AS2 siRNA6, sense: 5′-GCGAGCUGUCUGGCCUCAATT-3′ and antisense: 5′-UUGAGGCCAGACAGCUCGCTT-3′;

HKDC1 siRNA1, sense: 5′-CCAACGCCCAAUGAAAUCATT-3′ and antisense: 5′-UGAUUUCAUUGGGCGUUGGTT-3′;

HKDC1 siRNA2, sense: 5′-GAGCUUGUCAGGCUUAUCUTT-3′ and antisense: 5′-AGAUAAGCCUGACAAGCUCTT-3;

HKDC1 siRNA3, sense: 5′-CAGUGCGAAUGUACAACAATT-3′ and antisense: 5′-UUGUUGUACAUUCGCACUGTT-3′;

hsa-miR-876-5p agomir, sense: 5′-UGGAUUUCUUUGUGAAUCACCA-3′ and antisense: 5′-GUGAUUCACAAAGAAAUCCAUU-3′;

hsa-miR-876-5p antagomir: (antisense oligonucleotides): 5′-UGGUGAUUCACAAAGAAAUCCA-3′;

hsa-miR-3167 mimics, sense: 5′-AGGAUUUCAGAAAUACUGGUGU-3′ and antisense: 5′-ACCAGUAUUUCUGAAAUCCUUU-3′.

Western blot analysis

Protein samples were extracted using ice-cold lysis buffer (Beyotime Biotechnology, Shanghai, China) and then centrifuged at 13500 rpm for 15 min at 4 °C. Protein samples (30 μg) were separated and transferred onto nitrocellulose membranes. After a 1 h incubation with 5% nonfat milk, the membranes were incubated with primary antibodies, including anti-HKDC1 (Proteintech, 25874-1-AP, 1:500), anti-NLRP3 (Boster, BA3677, 1:500), anti-ASC (Bioss, bs-6741R, 1:500), anti-Caspase1 (Abcam, ab1872, 1:1000), anti-pro-Caspase1 (Abcam, ab179515, 1:1000) and anti-IL-1β (Abcam, ab200478, 1:500), overnight at 4 °C. Membranes were incubated with horseradish peroxidase-labeled secondary antibodies at room temperature and then visualized using an ECL detection kit (Beyotime Biotechnology, Shanghai, China).

Quantitative reverse transcription PCR (qRT–PCR)

Total RNA was extracted from EC tissues and Ishikawa and Hec-1-B cells using TRIzol reagent according to the manufacturer's instructions. Cytoplasmic and nuclear RNAs were isolated and purified using a

Norgen's Cytoplasmic & Nuclear RNA Purification Kit (#21000, Thorold, ON, Canada) according to the manufacturer's protocol. Around 1×10^7 cells were lysed with ice-cold lysis buffer, then cytoplasmic RNA and nuclear RNA were bound to the column separately in fractionation buffer and separated by RNA elution. First strand cDNA synthesis was performed using the superscript first-strand cDNA synthesis kit (Invitrogen), and then the cDNAs were quantified using SYBR Green real-time PCR. Primer sequences were as follows:

HOXC-AS2, forward: 5′-CAACTGCATGTGGCCTGTAG-3′ and reverse: 5′-GCAGGCCTTAGCTGGATTTG-3′;

GALE, forward: 5′-CCAGTACCTGCCCCTTGATG-3′ and reverse: 5′-GCACTGCGTTCCAAGTCTTG-3′;

HKDC1, forward: 5′-GGCAGATTTCATGAAGACCAAA-3′ and reverse: 5′-TCTTCCAGTTTAGTCTGTCGAC-3′;

GCNT3, forward: 5′-TGAGAAGACCAAGCTGACGC-3′ and reverse: 5′-CGTGGCAGCAAATGTGAACA-3′;

GALNT6, forward: 5′-TCGTCACCATCGACCTTAATAC-3′ and reverse: 5′-TTCTCATGTGGAGGAAGTGTTT-3′;

β-actin, forward: 5′- CTCACCATGGATGATGATATCGC-3′ and reverse: 5′-CACATAGGAATCCTTCTGACCCA-3′;

hsa-miR-876-5p, forward: 5′-GATGCTCTTGGATTTCTTTGTGA-3′ and reverse: 5′-TATGGTTGTTCACGACTCCTTCAC-3′; and

hsa-miR-3167, forward: 5′-ACAGGTGAGGATTTCAGAAATACTG-3′ and reverse: 5′-CAGAGCAGGGTCCGAGGTA-3′.

Figures:

Fig. S1

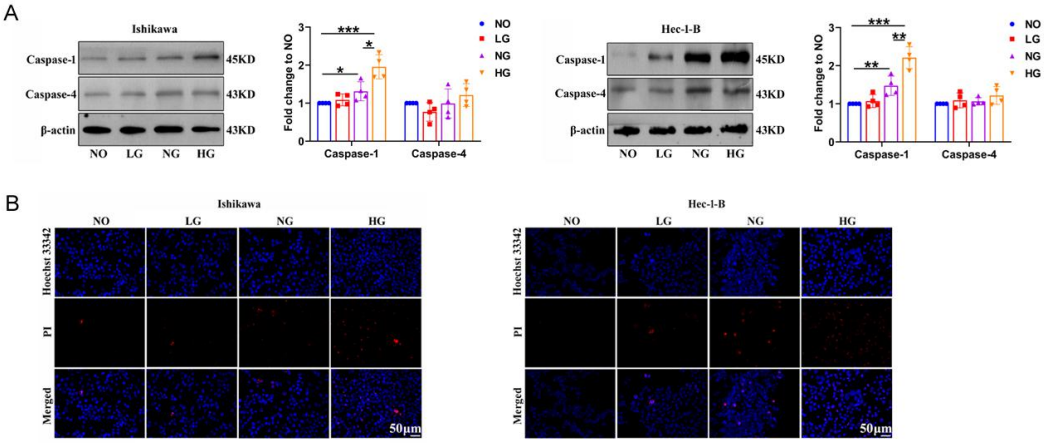

Fig. S1 Glucose promotes EC cell pyroptosis in a dose dependent manner. (A) Western blot analysis was used to detect Caspase1 and Caspase4 levels in Ishikawa and Hec-1-B cells treated with no glucose (NO), low glucose (1 mM, LG), normal glucose (5 mM, NG) and high glucose (25 mM, HG) (n=4). (B) Cell pyroptosis was assessed by PI staining assay (n=3). All values are presented as the means ± SD, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

Fig. S2

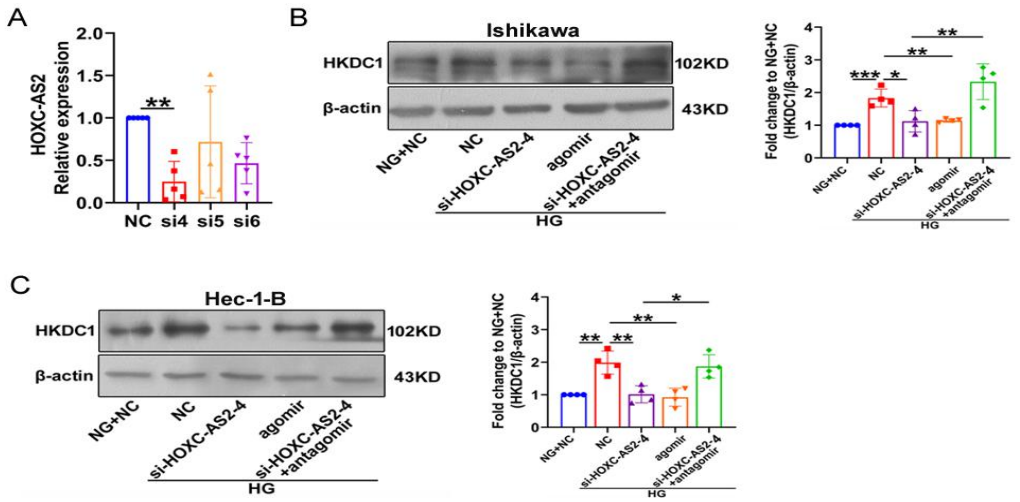

Fig. S2 (A) The interference efficiency of HOXC-AS2 siRNAs is shown, and si-HOXC-AS2-4 had the highest silencing efficienc (n=4). (B-C) The expression of HKDC1 was analyzed by Western blot assay in Ishikawa and Hec-1-B cells transfected with si-HOXC-AS2, miR-876-5p agomir and miR-876-5p antagomir (n=4). All values are presented as the means ± SD, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. NC, negative control; NG, normal glucose; HG, high glucose; agomir, miR-876-5p mimics; antagomir, miR-876-5p inhibitor; si-HOXC-AS2, small interfering RNA targeting HOXC-AS2.