Supplementary

Selective Expansion of Renal Cancer Stem Cells Using Microfluidic Single-cell Culture Arrays for Anticancer Drug Testing

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Author Contributions

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Contents：

Supplementary methods

RNA sequencing

Total RNA was extracted using TRIzol solution and transferred to an RNase-free EP tube. The cells were allowed to stand at room temperature for 10 minutes and then centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was carefully aspirated into another RNase-free EP tube, and 0.2 mL of chloroform was added and vigorously shaken for 15 seconds. The mixture was left at room temperature for 3 minutes and then centrifuged at 12000 rpm for 15 minutes at 4°C. The upper layer and water layer (400 μL) were collected, and an equal volume of isopropanol was added and left at room temperature for 10 minutes. The mixture was then centrifuged at 12000 rpm for 10 minutes, and the supernatant was discarded. The RNA pellet was washed with 1 mL of 75% ethanol at 4°C and centrifuged at 7500 g for 10 minutes. After drying, 25 μL of DEPC water was added. RNA quality was assessed by measuring the concentration using an ultraviolet spectrophotometer (Nanodrop, ThermoFisher), evaluating the integrity by non-denaturing agarose electrophoresis, and analyzing the total RNA using Agilent 2100 Bioenzymer.

To prepare the RNA for sequencing, single-stranded circular DNA molecules (ssCirDNA) are generated and assembled into a DNA nanosphere (DNB) containing over 200 copies. The resulting DNBs are then loaded onto a high-density DNA nano-chip, which is used to sequence the DNA using the combined probe anchor polymerization technique (cPP) to generate sequence reads of 50 bp/100 bp/150 bp. The sequencing data is processed using SOAPnuke (v 1.5.2) to filter out reads containing the sequencing adapter, reads with low mass basis ratio (base mass <= 5) greater than 20%, and reads with an unknown base ('N' base) ratio greater than 5%. The resulting CleanReads are stored in FASTQ format and aligned to the reference genome using HISAT2 (v 2.0.4). The expression level of each gene is calculated using RSEM (v

1.2.12) and a heat map is generated using phmap (v 1.0.8) to visualize gene expression across different samples. Differential expression analysis is performed using PositationDis with a False Discovery Rate (FDR) ≤ 0.001 and |Log2Ratio| ≥ 1. Genome Enrichment Analysis is used to determine if there is a significant difference between the experimental and control groups for predefined gene sets. Gene Collection Enrichment Analysis (GSEA, http://www.requirement.org/gline) is used to determine whether predefined gene sets (hallmark gene sets) differ significantly between the experimental and control groups.

Analysis of Gene Data Related to Stemness

The TCGA datasets (https://portal.gdc.cancer.gov) were used to obtain the expression level changes of these stemness-related genes. The data were analyzed using R Studio (version 4.0.1). To this end, we utilized the "survminer" and "survival" package. Specifically, we classified genes into high and low expression groups based on the best cut-off values. We then performed Kaplan–Meier curves to identify statistically significant angiogenesis pathway genes in patients with renal cancer.

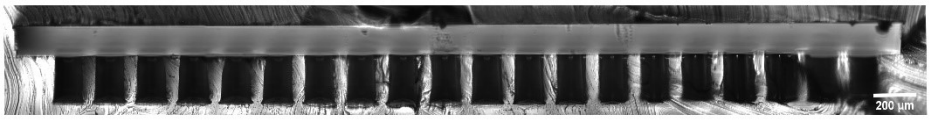

Figure S1 Side view of a single microfluidic module. Scale bar: 200 µm

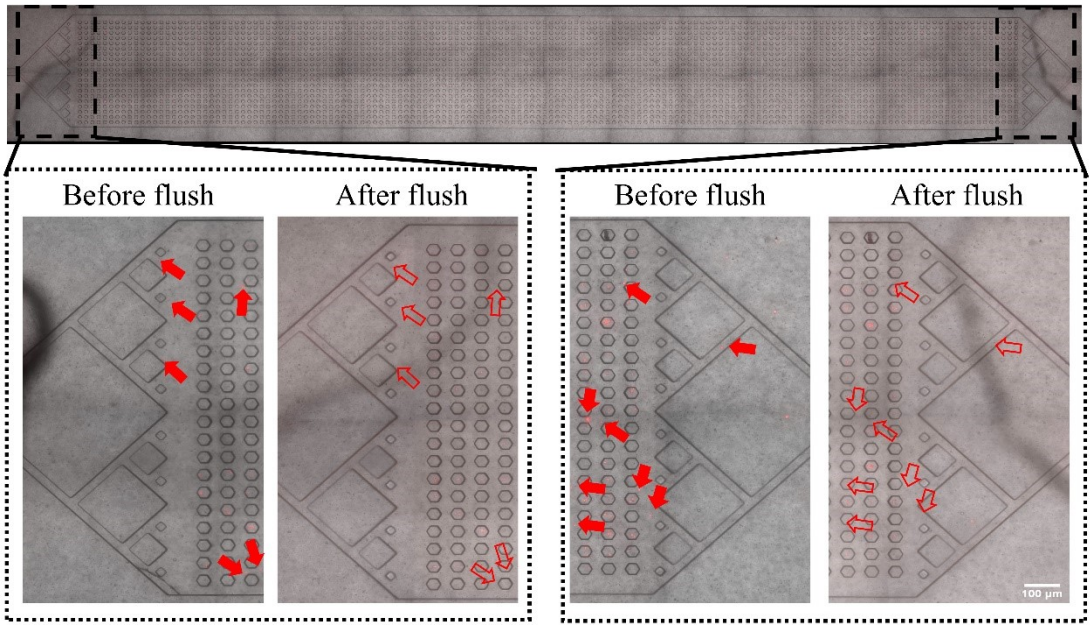

Figure S2 Cell culture medium flushing to eliminate residual cells. Photographs of the inlet and outlet regions were taken before and after flushing cell culture medium. After cell seeding, a limited number of cells remain trapped in the inlet/outlet channels. These uncaptured cells are subsequently flushed away by the flow of culture medium. Cells were stained with Celltracker Red. (Scale bars: 100 µm)

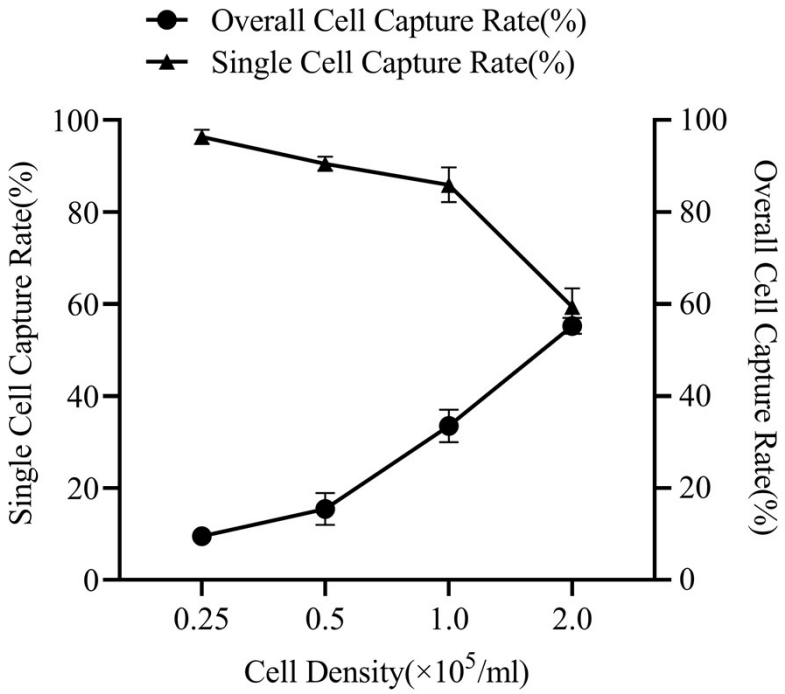

Figure S3 Cell occupancy in microwell arrays with various input cell densities.

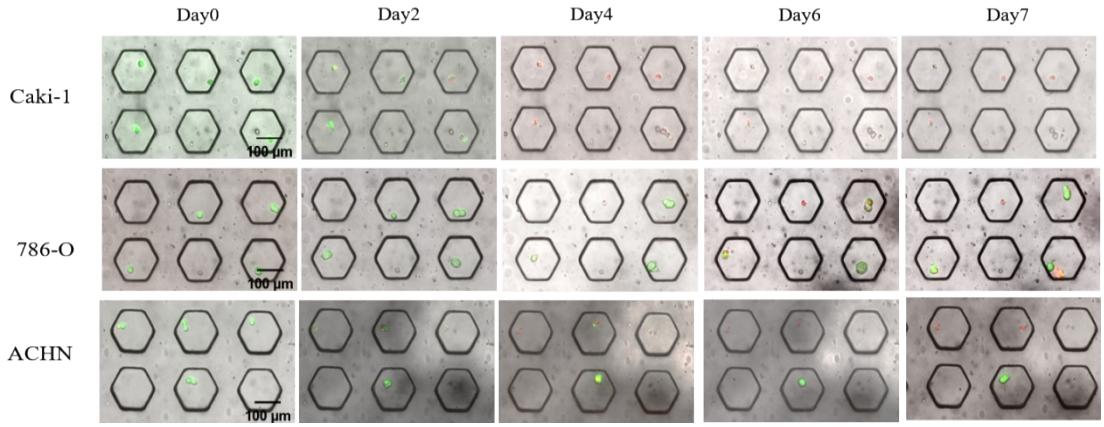

Figure S4 Selective expansion of single cells in microwell arrays. Only a fraction of the renal cancer cells survived and formed tumorspheres in the continuous single-cell culture. Cells were stained with CalceinAM/PI, with green fluorescence indicating live cells and red fluorescence indicating deceased cells. Scale bars:100 μm

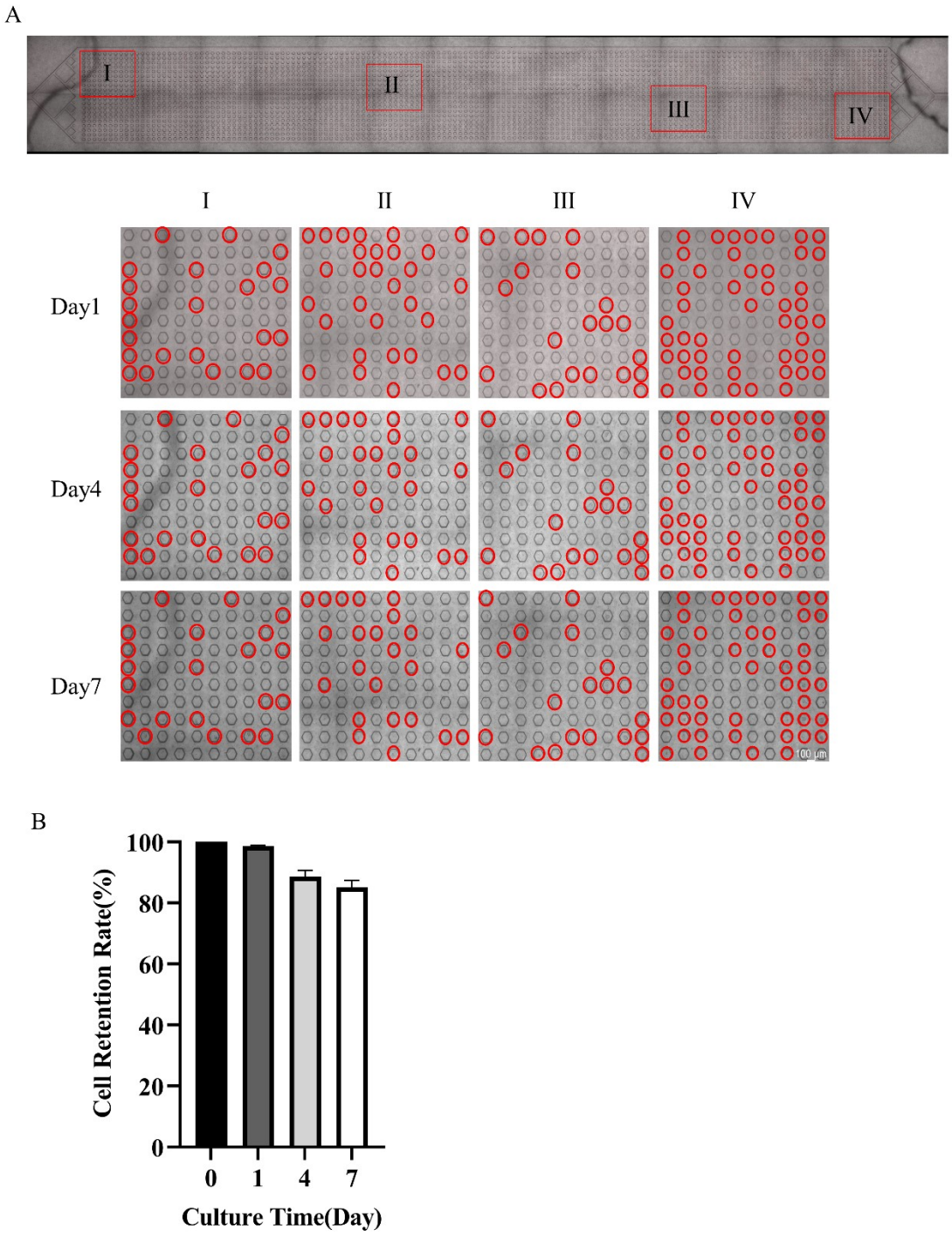

Figure S5 Images collected over days demonstrates cell retention in individual microwells. (A) Photographs taken on days 1, 4, and 7 show cell retention in various regions of the microwell array. The red circle indicates cell occupancy. Scale bar: 100 μm; (B) Cell retention rates determined on day1, day4 and day 7.

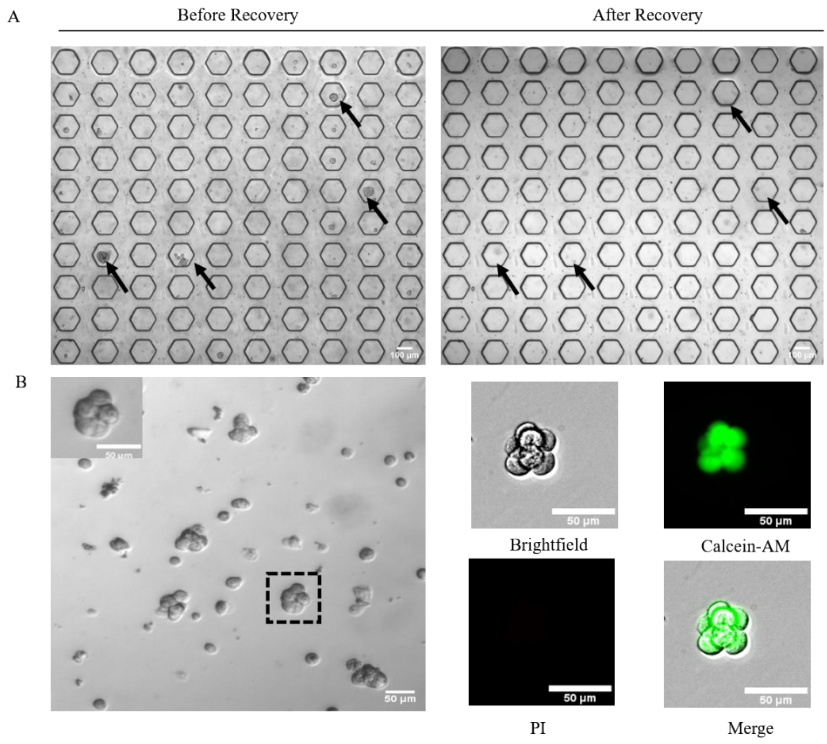

Figure S6 Cell recovery from the microfluidic chip. (A) Imaging of the microwell array before and after recovery demonstrates the discharge of cells from the microfluidic chip. Scale bars:100 μm. (B)Tumorspheres formed on the microfluidic chip were transferred to a petri dish, and the viability of cells within the tumorspheres was indicated through Calcein-AM/PI staining. Scale bars:50 μm.

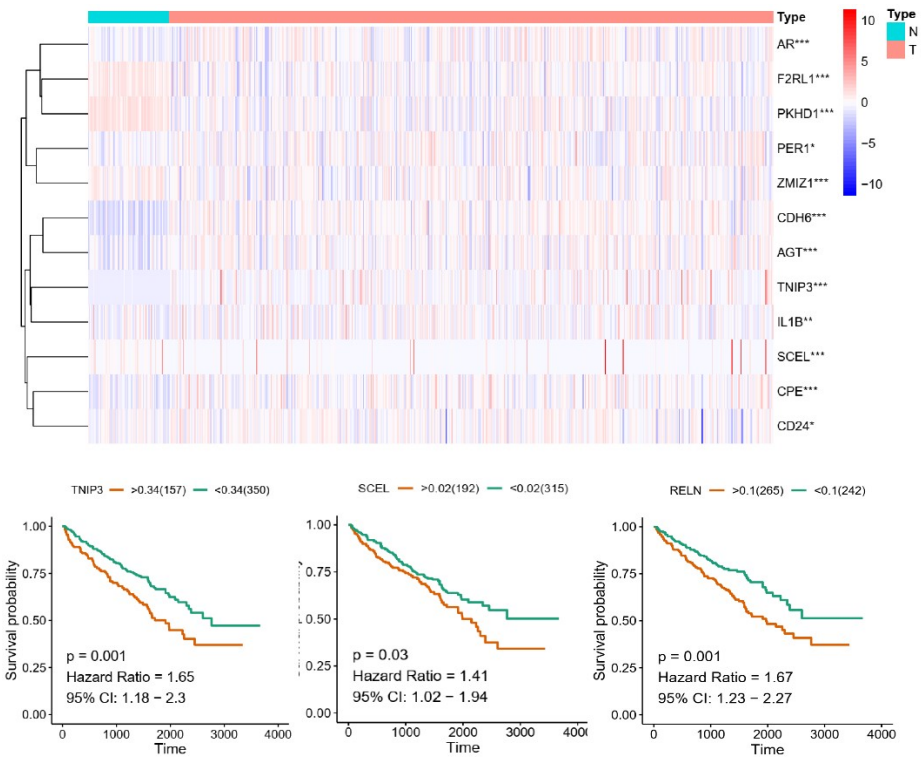

Figure S7 Clinical correlations of renal cancer with the expressions of stemness-related genes. (A) Comparison of the expression of stemness-related genes between cancer tissue cohorts and normal tissue cohorts in the TCGA database. (B) The survival curves of stemness-related genes associated with poor prognosis in renal cancer were analyzed based on their expression levels and clinical survival information in the TCGA samples. *:P<0.05, **: P<0.01, ***: P<0.001.

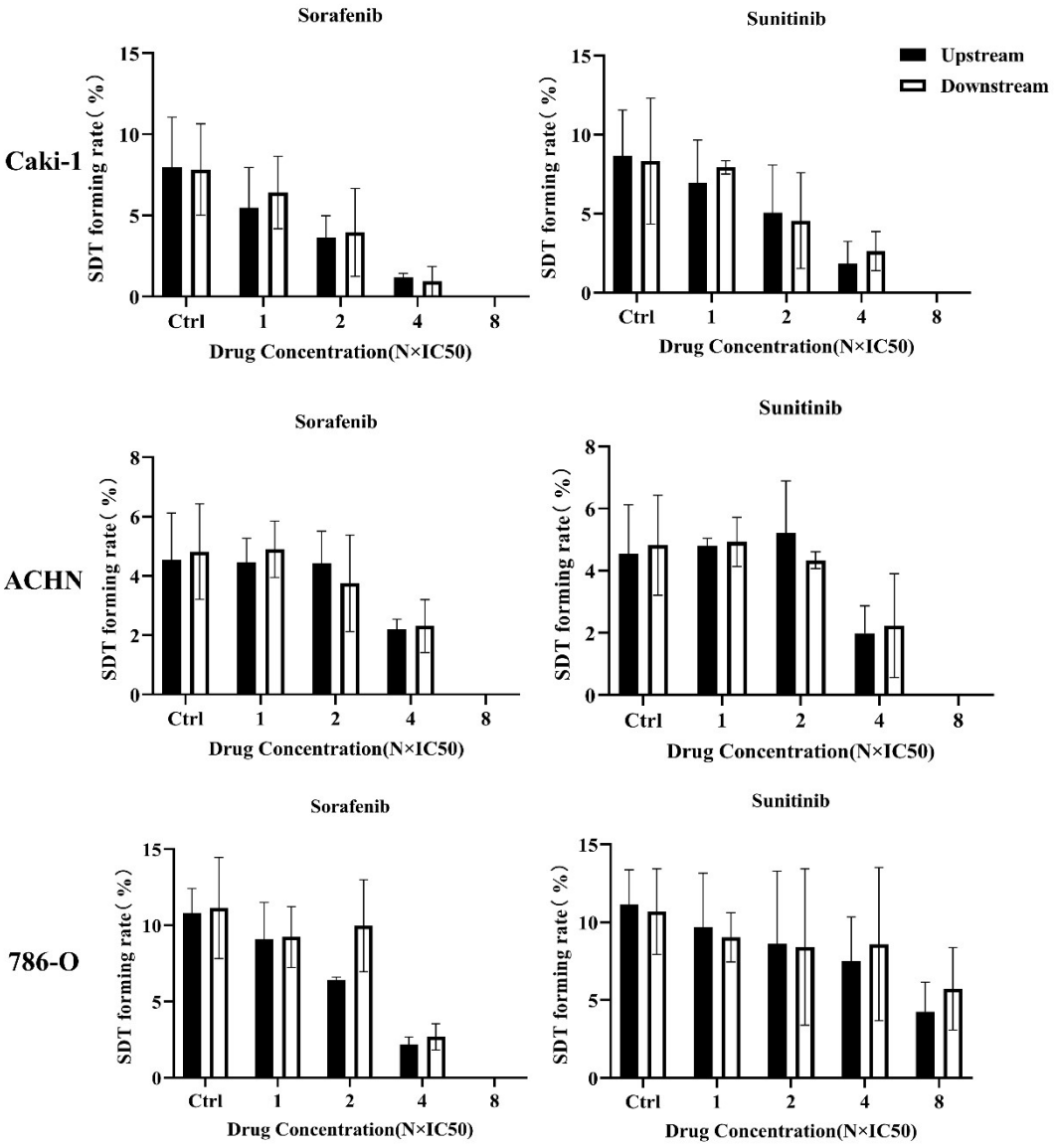

Figure S8 Comparison of SDT formation rates in upstream and downstream of the microwell array. The SDT formation rates of Caki-1 ACHN, and 786-O cells in upstream and downstream regions were assessed separately. Statistical analysis (*t-test*) shows no significant difference in SDT formation rates between upstream and downstream regions (p=0.0685, p=0.179).

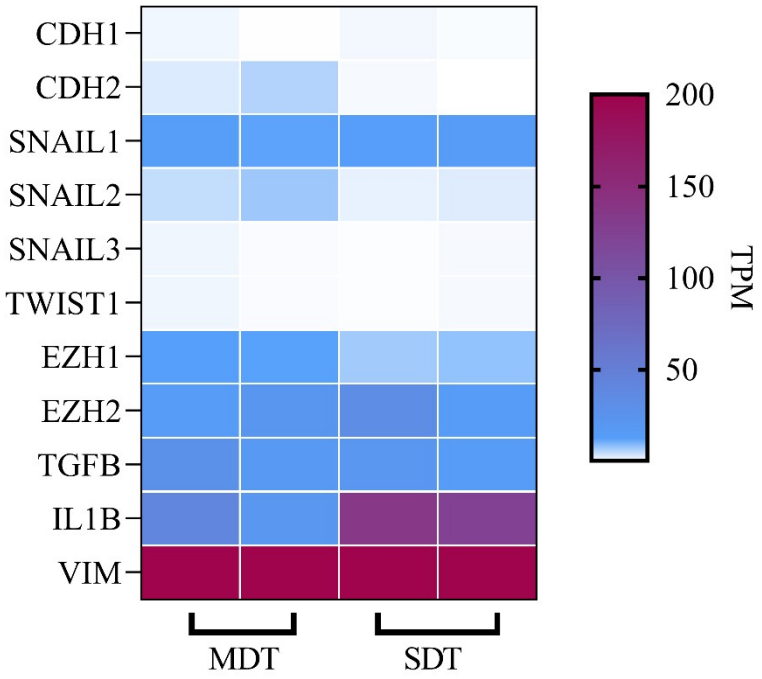

Figure S9 Expressions of EMT-related genes in the MDT and SDT cells. In the SDT group, epithelial-related genes CDH1 and CDH2 were down-regulated, while mesenchyme-related genes SNAIL1, EZH1, EZH2, IL1B, and VIM were up-regulated.