Manipulating Macrophage Polarization with Nanoparticles to Control Metastatic Behaviour in Heterotypic Breast Cancer Microtissues via Exosome Signalling

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Supporting Information

Section 1. Investigation of the cytotoxicity of nanoparticles

To examine the potential cytotoxic effects of nanoparticles on mouse macrophages (Raw264.7), nanoparticles were first applied to the macrophage culture in two different doses, and then inverted phase contrast images were captured (Figure S1).

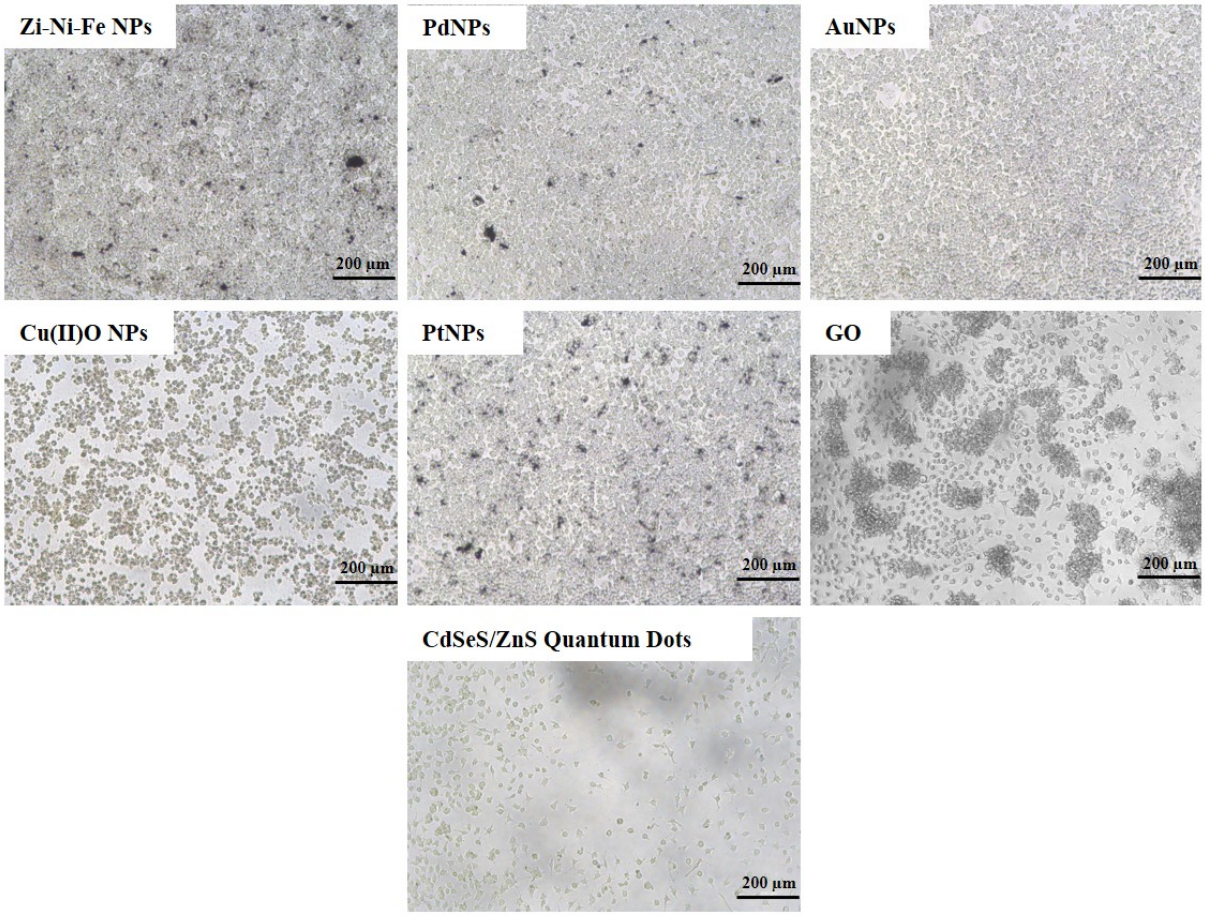

Figure S1. Microscopic assessment of the effects of nanoparticles on the proliferation of Raw264.7 cells

Across the tested nanoparticles, PtNPs were identified with their low toxicity and high potential for M1 polarization of macrophages. Thus, further analysis for their cell compatibility was conducted by a live-dead assay (Figure S2A,B). To this end, macrophages treated with PdNPs (50 µg/ml) were stained with Calcein-AM (green, stains live cells) and ethidium homodimer I (red, stains dead cells red). Untreated cells were used as negative control. To further investigate cytocompatibility by a cell proliferation test, we conducted an XTT assay for macrophages that were treated or untreated with PdNPs (50 µg/ml).

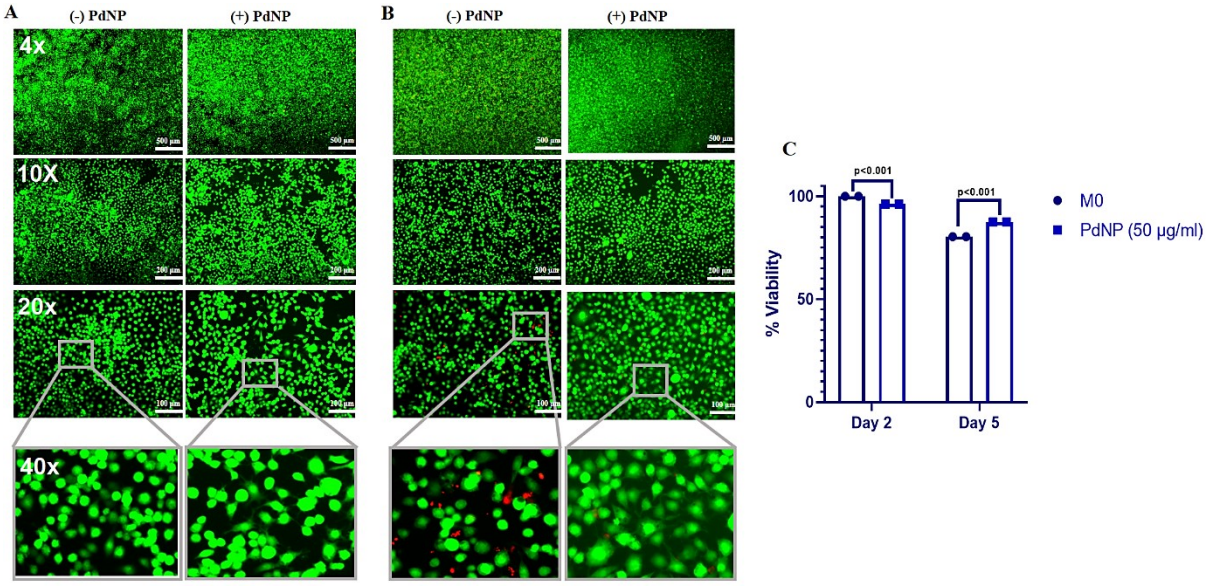

Figure S2. Cytocompatibility of PtNPs. Fluorescent microscopy images related to Calcein-AM (green, stains live cells) and ethidium homodimer-I (red, stains death cells) staining of macrophages, captured at day 2 (A) and day 5 (B). (C) Cell proliferation test applied in PdNPs (50 µg/ml) treated Raw264.7 cells (square) and untreated Raw264.7 cells as control (circle). The groups were compared by t-test.

Section 2. Investigation of the effects of exosomes on drug response

To investigate the effects of exosomes on drug resistance in tumor micro-tissues, MCF-7 micro-tissues, MCF-7/HUVEC micro-tissues, and MCF-7/HUVEC/phDFs micro-tissues were treated with PdNPs-Exo, M0-Exo, M1-Exo, and M2-Exo, followed by the application of 5-FU (10 and 100 µM). Microscopic images were captured and presented in Figure S3-5.

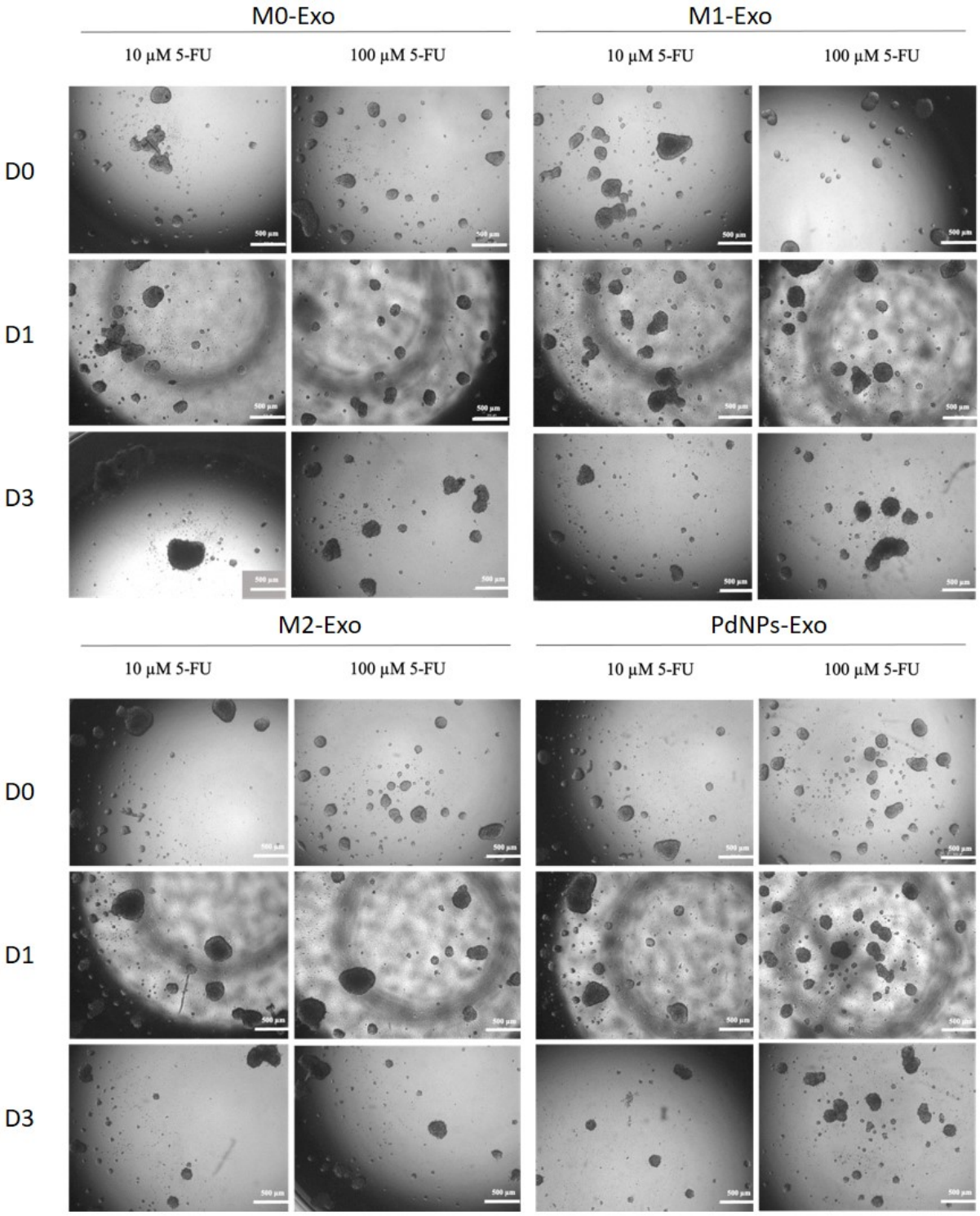

Figure S3. Inverted phase-contrast microscope images showing the effects of PdNPs-Exo, M0-Exo, M1-Exo, and M2-Exo on the response of MCF-7 micro-tissues to the applied drug.

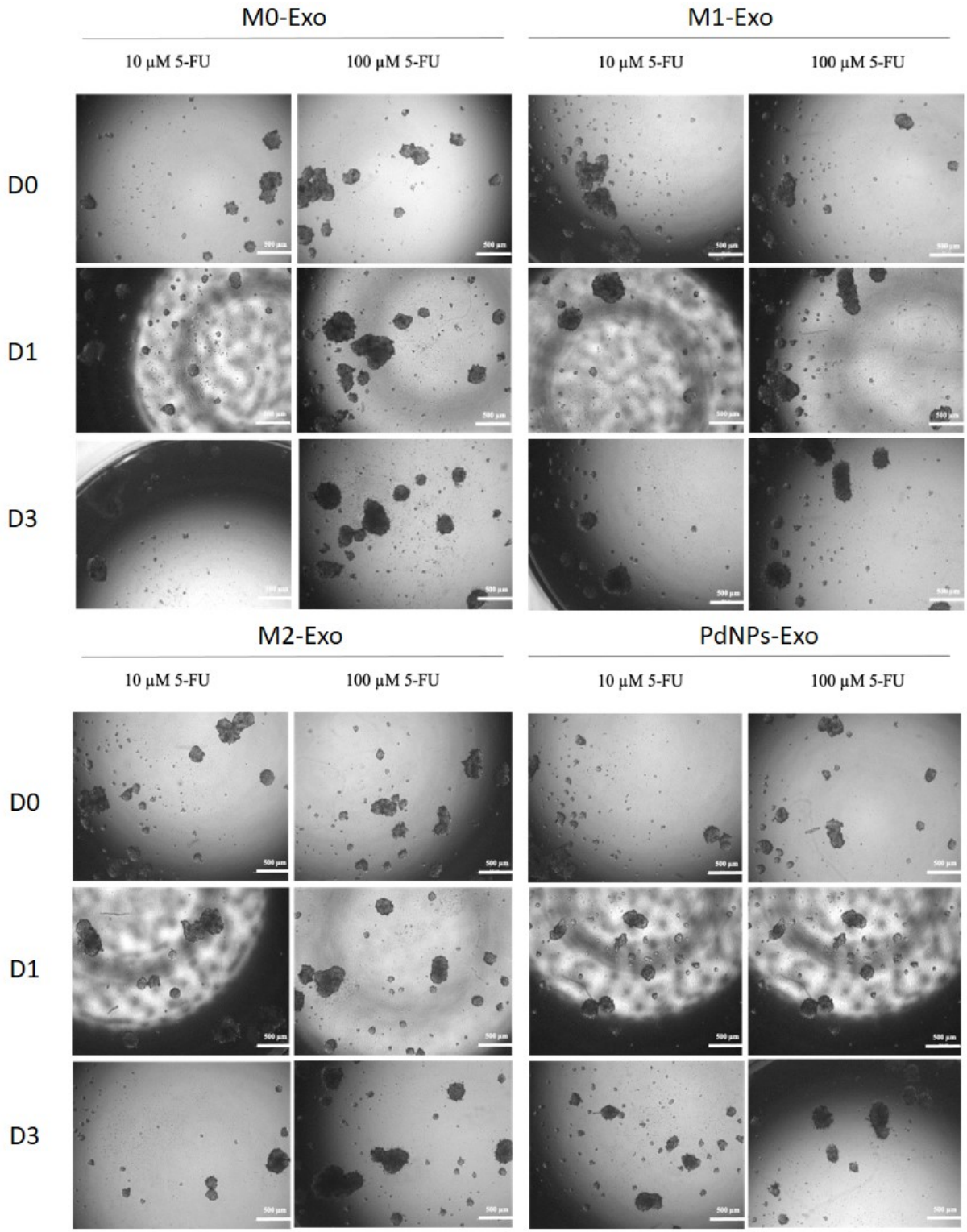

Figure S4. Inverted phase-contrast microscope images showing the effects of PdNPs-Exo, M0-Exo, M1-Exo, and M2-Exo on the response of MCF-7/HUVECs tumour micro-tissues to the applied drug.

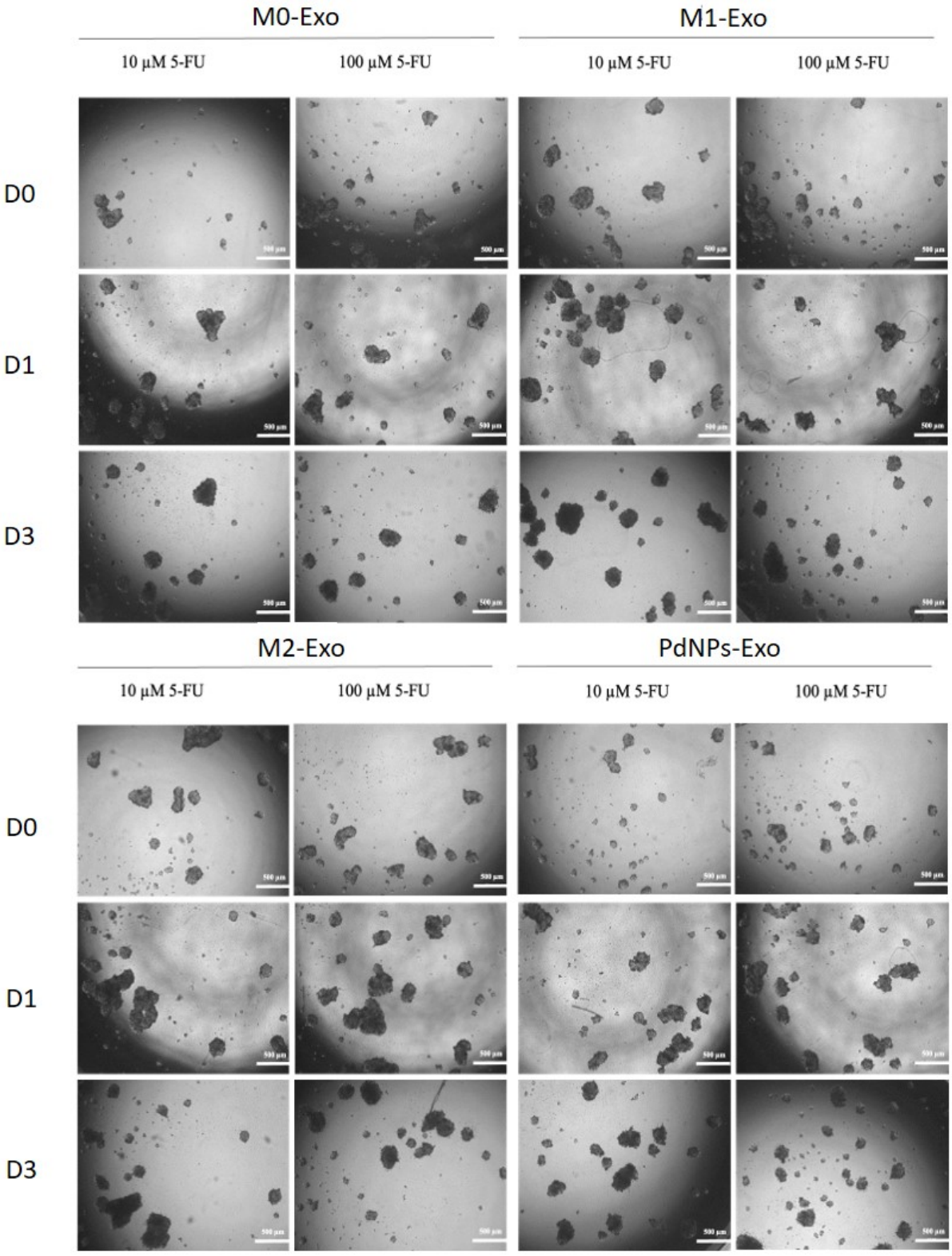

Figure S5. Inverted phase-contrast microscope images showing the effects of PdNPs-Exo, M0-Exo, M1-Exo, and M2-Exo on the response of MCF-7/HUVECs/phDFs micro-tissues to the applied drug.

Section 3. Protocol for Proteome Analysis

A total of 50 μg of protein from each sample (M0-Exo, M1-Exo, and PdNPs-Exo) was carefully transferred to microtubes. The proteins were first reduced with dithiothreitol (DTT) to attain a final concentration of 5 mM at 56 ºC. Subsequently, iodoacetamide (IAA) was added to achieve a final concentration of 50 mM, allowing for protein alkylation at room temperature for 1 hour in the dark. To further optimize the reduction process, additional DTT was introduced, reaching a final concentration of 10 mM. In order to precipitate the proteins, a methanol/chloroform precipitation protocol was employed. The resulting precipitate was then dissolved in a solution containing 8 M urea and 50 mM Tris buffer at pH 8.5. To attain a suitable urea concentration, the solution was subsequently diluted to 1 M using 50 mM Tris buffer at pH 8.5.

For enzymatic digestion, trypsin dissolved in 50 mM Tris buffer at pH 8.5 was added to the protein solution at a ratio of 1:100 (w/w, trypsin-LysC/protein). The mixture was incubated overnight to ensure complete digestion. To halt the enzymatic activity, trifluoroacetic acid was added, setting the final concentration at 0.5% (v/v). To purify the samples, a Sep-Pak purification system (Waters, UK) was employed following the manufacturer's protocol. The purified samples were then dried using a Speed-Vac centrifugal evaporator and subsequently reconstituted in MilliQ water (18.2 MΩ·cm) to achieve a protein concentration of 1 μg/mL.

Peptides were separated using an EASY-Spray column [50 cm length, 75 μm internal diameter (ID), PepMap C18, 2 μm particles, and 100 Å pore size]. The analysis was performed on an Ultimate 3000 RSLnano system (Dionex, Thermo Scientific) connected to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Germany).

To load the samples onto the column, buffer A (0.1% formic acid in water) was used, and elution was carried out using a 150-minute gradient with buffer B (95% acetonitrile, 0.1% formic acid), starting from 5% and reaching 95% over the course of the gradient. The flow rate was set at 250 nL/min. Mass spectra were acquired using an Orbitrap Q Exactive Plus mass spectrometer in data-dependent mode, scanning the range from 500 to 2000 m/z over the 150-minute run. Peptide fragmentation was performed through higher-energy collisional dissociation (HCD) with an energy setting of 29 normalized collision energy (NCE). The MS/MS spectra were acquired at a resolution of 17,500.

For data analysis, the output data from the MS analysis were processed using MaxQuant (ver. 2.1.4.0). Proteins were identified using the UniProt Human Database in the presence of the potential contaminants *(Taxonomy ID: 9606)*. Methionine oxidation and acetylation at protein N termini were specified as variable modifications, and carbamidomethylation of cysteine as

fixed modification. Apart from default search parameters, Acetylation at N termini and Oxidation (P) were included in the variable modification and label-free quantification (LFQ) was enabled. Trypsin and LysC were selected as the digestion enzyme and the maximum missed cleavages were determined two. The data were searched against the UniProt database (Taxonomy ID: 9606-Homo sapiens). The search range was limited to a peptide length of 7 amino acids and a maximum peptide mass of 4600 Da. The false discovery rate (FDR) for peptide spectrum matches and protein identifications was set at less than 1% using a target-decoy approach as in the default settings. Post-processing was performed in Perseus software (ver. 2.0.7.0). Potential contaminants, reverse-sequence proteins, and proteins identified "only by site" were filtered in addition to proteins that are only identified by a single peptide as well as proteins that contain higher than %30 missing values in total. All LFQ intensities were converted to a $\log_2$ scale and normalized by the subtracting algorithm. Missing values were imputed to create a normal distribution with left-shifted by 1.8 and a width of 0.3.