Modulation of anti-angiogenic activity using ultrasound-activated nutlin-loaded piezoelectric nanovectors

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1. Introduction

Angiogenesis is the process referred to the vessel growth, but most usually to a new vessel formation from the already existing vasculature. It plays an essential role in many processes such as embryonic development, ovulation, and wound healing; moreover, it is critical in the progression of many diseases (including arthritis and cancer metastasis) [1, 2]. Survival of metastatic cells and thus tumor progression require nutrients and an adequate supply of oxygen; hence, metastatic cells need to stay near blood capillaries for direct contact to the circulatory system [3]. Tumor cells and tumor-associated stromal cells induce an angiogenic switch by the constant secretion of pro-angiogenic factors and stimulate endothelial cell proliferation/migration to generate new blood vessels during cancer progression. In this process, the increased number of endothelial cells leads to form disorganized and immature vessels with disrupted endothelial cell junctions, associated with tumor neo-vessels permeability, fragility, and interstitial fluid pressure [4].

Although tumor angiogenesis is considered a primary therapeutic target, it is also a major challenge in cancer medicine. Conventional therapies focus on the application of anti-angiogenic agents to target and then block the activity of pro-angiogenic factors [5]. Novel approaches envision the combination of anti-angiogenic drugs with chemotherapy or immunotherapy; however, their effectiveness remains a matter of debate [6]. On the other side, nanotechnology offers great tools to target and release anti-angiogenic agents in specific diseased areas. In this work, we showed that the angiogenic behavior of human cerebral microvascular endothelial cells can be inhibited by using nutlin-3a-loaded ApoE-functionalized polymeric piezoelectric nanoparticles, which can remotely respond to ultrasound stimulation. The anti-angiogenic effect, derived from the use of chemotherapy and chronic piezoelectric stimulation, leads to disruption of tubular vessel formation, decreased cell migration and invasion, and inhibition of angiogenic growth factors in the presence of migratory cues released by the tumor cells. Overall, the proposed use of remotely activated piezoelectric nanoparticles could provide a promising approach to hinder tumor-induced angiogenesis.
cancer progression upon US stimulation by activating anti-proliferation and apoptosis pathways, inducing necrosis, inhibiting cell migration, and decreasing invasion in drug-resistant glioblastoma cells [15].

In the present work, we prepared piezoelectric lipid-polymer nanoparticles owning a core of P(VDF-TrFE) and loaded with nutlin-3a (Nut), being the anti-angiogenic activity of Nut widely reported in the literature [16,17]. The particles were further functionalized with a peptide composed of specific residues of apolipoprotein E (ApoE), that interacts with caveolin-1 protein in endothelial cells [18]. Human cerebral microvascular endothelial cells (hCMEC/D3) were used being widely exploited as a component of blood-brain barrier (BBB) in vitro models: they are in fact particularly suitable for the investigation of cellular and molecular mechanisms at the base of many pathologies associated to the central nervous system, including brain cancer [19]. The prepared system was used to modulate the angiogenesis upon US stimulation: in vitro studies showed the potential inhibitory effect of the particles on vessel formation, on endothelial cells migration and invasion, and on production of angiogenesis-related cytokines. This study, for the first time in the literature, shows the interaction of piezoelectric nanoparticles with human endothelial cells, and their effect, following mechanical stimulation, on the inhibition of tumor-induced angiogenesis.

2. Experimental Section

2.1. Nanoparticle fabrication and characterization

Nutlin-3a loaded P(VDF-TrFE) nanoparticles (Nut-PNPs) were synthesized and surface-functionalized with a peptide that corresponds to a fragment of apolipoprotein E (ApoE; GenScript), as previously described in a work of our group [15]. Briefly, 2 mL of 5 mg/mL P(VDF-TrFE) (45:65; Piezotech) and 200 μL of 5 mg/mL nutlin-3a (Sigma-Aldrich) in acetone (Sigma-Aldrich) were placed into 4.5 mL of a 1 mg/mL 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-poly(ethylene glycol) (DSPE-PEG; Nanocs) dispersion in water under stirring. The obtained mixture underwent three consecutive cycles of sonication (ultrasonic tip; Fisherbrand™ Q125 Sonicator) and purification (Amicon® centrifuge filters, Ultra-4 Centrifugal Filter Unit, 100 kDa; Sigma-Aldrich) with the pellet suspended in water (4 mL). The polymeric core of the nanoparticles was recrystallized by refluxing (90 °C for 60 min) and then by progressively cooling down to 25 °C (−1 °C/min). Subsequently, 1 mg of DSPE-PEG/DSPE-PEG-maleimide (1:1) was added for the stabilization of the nanoparticle dispersion, which was sonicated for 10 min (ultrasonic tip, 70% amplitude; Fisherbrand™ Q125 Sonicator). Finally, three consecutive centrifugation steps were performed at 15 °C (2460 g for 15 min; Amicon® centrifuge filters, Ultra-4 Centrifugal Filter Unit, 100 kDa; Sigma-Aldrich) to remove the excess of lipids. The bioconjugation of the nanoparticles with the ApoE peptide was carried out through the maleimide-thiol click reaction [20] by incubating the 141–150 residues of the ApoE (200 μL of a 2 mg/mL water suspension) to 4 mL of the 2 mg/mL nanoparticle dispersion for 4 h at 4 °C under shaking. Three centrifugation steps were then carried out as described above to remove the non-bounded peptide. ApoE-PNPs were synthesized following the same protocol used for ApoE-Nut-PNPs, without adding the drug in the polymer/acetone solution. Nut-PNPs and PNPs were obtained skipping the functionalization step. The fluorescent staining of the nanoparticles was obtained by adding 5 μL of fluorescent dye (Vybrant™; Invitrogen) to the 2 mL polymer/acetone initial solution.

Morphologic analysis of PNPs, Nut-PNPs, ApoE-PNPs, and ApoE-Nut-PNPs was performed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Concerning TEM, a drop of 70 μg/mL nanoparticle dispersions was cast on an ultrathin amorphous carbon film-coated Cu grid composed of 150 mesh and, subsequently, imaging was performed with a JEOL 1011 operating at 100 kV. For SEM imaging, a drop of nanoparticle dispersions was deposited on a silicon wafer. Then, samples were imaged with a Helios NanoLab 600i Dual BeamTM FIB/SEM FEI after gold-sputtering (30 mA for 1 min) with a Quorum Tech Q150RES Gold Sputter Coater. The nanoparticle size was measured from the TEM images by using ImageJ software, and data were reported as average diameter ± standard deviation.

The hydrodynamic size of PNPs, Nut-PNPs, ApoE-PNPs, and ApoE-Nut-PNPs (500 μg/mL) were investigated in water at 37 °C by using a Nano Z-Sizer 90 (Malvern Instrument); the ζ-potential measurements were performed in the same conditions. The hydrodynamic size and ζ-potential measurements are shown as the mean ± standard deviation of three different measurements with 10 runs for each of them. CONTIN analysis was used to obtain the intensity distribution, and the value of the hydrodynamic diameter and the polydispersity index (PdI) was assessed by cumulant analysis. Furthermore, stability of PNPs, Nut-PNPs, ApoE-PNPs, and ApoE-Nut-PNPs was assessed at a concentration of 500 μg/mL in plasma obtained from mice blood (see Section 2.4 for details), at 37 °C for 14 days, periodically performing dynamic light scattering measurements.

2.2. Cell culture

Human cerebral microvascular endothelial cells (hCMEC/D3; Merck Millipore) were cultured in EndoGro-MV (Sigma-Aldrich) supplemented with EndoGro-MV-VEGF Complete Culture Media Kit (SCM0003; Sigma-Aldrich), and 1% penicillin-streptomycin (Gibco). T98G cells (ATCC® CRL-1690™) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1 mM sodium pyruvate (Gibco), 2 mM L-glutamine (Gibco), and 100 IU/mL of penicillin-streptomycin (Gibco). All cell cultures were maintained at 37 °C in a 5% CO2 atmosphere.

2.3. WST-1 assay

For proliferation analysis, hCMEC/D3 cells were seeded at 2·10⁴ cells/cm² density on Matrigel (10 mg/mL; BD Bioscience) coated 96-well plates and analyzed using WST-1 assay reagent (BioVision) following the manufacturer’s instruction. After 24 h from cell seeding, they were incubated with PNPs (100–1000 μg/mL), Nut-PNPs (100–1000 μg/mL), ApoE-PNPs (100–1000 μg/mL), ApoE-Nut-PNPs (100–1000 μg/mL), or Nut (0.2–25 μM) for 72 h. After rinsing the cells with PBS, they were treated with the WST-1 reagent (1:10 dilution in complete EndoGro-MV medium for 45 min at 37 °C). The absorbance of the collected supernatants was measured using a microplate reader (Perkin Elmer Vector X3) at 450 nm. The absorbance of blank, corresponding to EndoGro-MV medium, was subtracted from all measurements. The results were then normalized with respect to the non-treated control culture.

2.4. Hemolysis test

The effect of the nanoparticles on red blood cells (RBCs) integrity was evaluated by a standard hemolysis assay with some modifications [21]. Briefly, blood was collected from mice (discharged samples obtained from animals sacrificed at the end of experimental procedures approved by the ethical committee, authorization 746/2021-PR of the Italian Ministry of Health), and added to a 3.8% sodium citrate solution to prevent coagulation. It was then mixed by gentle inversion of the tube and centrifuged at 1000 g for 10 min. The plasma supernatant was removed, and the RBCs were washed 3 times using saline solution (0.9% w/v). The final suspension consisting of 5% (v/v) RBCs was obtained by adding saline solution. 500 μg/mL of PNPs, Nut-PNPs, ApoE-PNPs, or ApoE-Nut-PNPs were added in 5% (v/v) RBC suspension and incubated for 72 h at 37 °C by gently shaking on an orbital plate shaker. The positive control (C+) consisted of 6% (v/v) PBS solution to prevent coagulation. It was then mixed by gentle inversion of the tube and centrifuged at 1000 g for 10 min. The plasma supernatant was removed, and the RBCs were washed 3 times using saline solution (0.9% w/v). The final suspension consisting of 5% (v/v) RBCs was obtained by adding saline solution. 500 μg/mL of PNPs, Nut-PNPs, ApoE-PNPs, or ApoE-Nut-PNPs were added in 5% (v/v) RBC suspension and incubated for 72 h at 37 °C by gently shaking on an orbital plate shaker. The positive control (C+) consisted of 6% (v/v) PBS solution to prevent coagulation. It was then mixed by gentle inversion of the tube and centrifuged at 1000 g for 10 min. The plasma supernatant was removed, and the RBCs were washed 3 times using saline solution (0.9% w/v). The final suspension consisting of 5% (v/v) RBCs was obtained by adding saline solution. 500 μg/mL of PNPs, Nut-PNPs, ApoE-PNPs, or ApoE-Nut-PNPs were added in 5% (v/v) RBC suspension and incubated for 72 h at 37 °C by gently shaking on an orbital plate shaker. The positive control (C+) consisted of 6% (v/v) PBS solution to prevent coagulation. It was then mixed by gentle inversion of the tube and centrifuged at 1000 g for 10 min. The plasma supernatant was removed, and the RBCs were washed 3 times using saline solution (0.9% w/v). The final suspension consisting of 5% (v/v) RBCs was obtained by adding saline solution.
positive controls (100% hemolysis), respectively. The percentage of hemolysis was calculated normalizing all experimental results to the mean absorbance value, which represents 100% hemolysis in positive control.

2.5. Cellular internalization

Cells were seeded at 2 \times 10^4 cells/cm² in μ-Dishes (35 mm; Ibidi) coated with Matrigel (10 mg/mL) for confocal microscopy imaging. After 24 h, cultures were incubated with 500 μg/mL of DIO-labelled PNP, Nut-PNPs, ApoE-PNPs, or ApoE-Nut-PNPs for further 24 and 72 h. Then, the cells were fixed using 4% paraformaldehyde (PFA; Sigma-Aldrich) at 4°C for 20 min. Next, they were rinsed three times with PBS. hCMEC/D3 cells were incubated with TRITC-phalloloid (1:200 v/v; Sigma-Aldrich) and Hoechst (1:1000 v/v; Invitrogen) at 37°C for 45 min for the imaging of nuclei and f-actin, respectively. Finally, a confocal fluorescence microscope (C2 system Nikon) was used to acquire 2D images and 3D rendering.

2.6. In vitro tube formation assay

Tube formation assay was carried out by growing hCMEC/D3 cells on Matrigel-coated wells. A 24-well plate was coated with Matrigel (10 mg/mL), allowed to solidify at 37°C for 30 min. Cell cultures were divided in 8 groups including controls w/o (Control) or w/ (Control + US) US stimulation, cells treated with free w/o (Nut) or w/ (Nut + US) US stimulation, cells treated with ApoE-PNPs w/o (ApoE-PNPs) or w/ (ApoE-PNPs + US) US stimulation, and cells treated with ApoE-Nut-PNPs w/o (ApoE-Nut-PNPs) or w/ (ApoE-Nut-PNPs + US) US stimulation. To assess the particle effect on the tube formation, cells (2 \times 10^4 cells/cm²) were treated for 24 h with Nut 21.5 μM (corresponding to Nut loaded in 500 μg/mL of ApoE-Nut-PNPs), ApoE-PNPs (500 μg/mL), or ApoE-Nut-PNPs (500 μg/mL). A previously described protocol was followed for chronic US stimulation [15]. Briefly, the US was delivered at 1 MHz frequency and 1 W/cm² intensity. Single stimuli lasted 200 ms each and were activated every 2 s. US were applied for 1 h for 2 days. This protocol was used to measure the protein content, according to the manufacturer's protocol, by exploiting standard calibration curves (Fig. S1B) and normalizing the values with respect to the total protein content per sample. For protein extraction, 8 M urea (Sigma-Aldrich) and protease inhibitor (1:1000 v/v, Sigma-Aldrich) were used to lyse the cells. Finally, the bicinchoninic acid assay (BCA; Sigma-Aldrich) was used to measure the protein content, according to the manufacturer's protocol.

2.9. Cytokines release assessment

hCMEC/D3 cells (2 \times 10^4 cells/cm²) were incubated with plain medium (as control), Nut (21.5 μM), ApoE-PNPs (500 μg/mL), or ApoE-Nut-PNPs (500 μg/mL) for 24 h on Matrigel-coated wells. After incubation, cultures were stimulated with US as previously described (1 h per day for 2 days). The supernatants were then collected and centrifuged at 1000 g for 10 min for the elimination of any cellular debris. In order to profile angiogenesis-related cytokines, i.e., tumor necrosis factor-alpha (TNF-α), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), basic fibroblast growth factor (FGF), transforming growth factor beta (TGFβ), epidermal growth factor (EGF), and leptin, human angiogenesis ELISA strip1 (Signosis) was performed following the manufacturer's protocol, by exploiting standard calibration curves (Fig. S1B) and normalizing the values with respect to the total protein content per sample. For protein extraction, RIPA buffer (Sigma-Aldrich) and protease inhibitor (1:1000 v/v, Sigma-Aldrich) were used to measure the protein content, according to the manufacturer's protocol.

2.10. Statistical analysis

Statistical analysis was carried out using analysis of variance (ANOVA) followed by Fisher's post-hoc test; data are presented as mean ± standard deviation of three independent experiments. The significance was set at p < 0.05.
3. Results

3.1. Nanoparticle characterisation

The characterization of PNPs, Nut-PNPs, ApoE-PNPs, and ApoE-Nut-PNPs is reported in Fig. 1 and Fig. S2. Representative TEM and SEM images of ApoE-PNPs (Fig. 1A and C), ApoE-Nut-PNPs (Fig. 1B and D), PNPs (Fig. S2A and Fig. S2C), and Nut-PNPs (Figs. S2B and S2D) suggest the presence of spherical-shaped nanoparticles. The size of the PNPs, Nut-PNPs, ApoE-PNPs, and ApoE-Nut-PNPs, estimated through image analysis, resulted to be respectively 62 ± 20 nm, 56 ± 12 nm, 66 ± 22 nm, and 76 ± 16 nm. The hydrodynamic diameters of PNPs, Nut-PNPs, ApoE-PNPs, and ApoE-Nut-PNPs in water resulted instead to be respectively 212 ± 10 nm, 266 ± 5 nm, 267 ± 2 nm, and 203 ± 2 nm (Fig. 1E and Fig. S2E), with a PDI of 0.32 ± 0.08, 0.30 ± 0.09, 0.33 ± 0.05, and 0.18 ± 0.01: as expected, these values are slightly higher than the particle size determined with electron microscopy due to the presence of the hydration shell. The ζ-potential of PNPs, Nut-PNPs, ApoE-PNPs, and ApoE-Nut-PNPs resulted −20.8 ± 0.9 mV, −18.4 ± 0.8 mV, −21.6 ± 0.7 mV, and −18.3 ± 0.6 mV, respectively (Fig. 1F and Fig. S2F). The stability of PNPs, Nut-PNPs, ApoE-PNPs, and ApoE-Nut-PNPs was monitored using DLS measurements up to 14 days (Fig. 1G and Fig. S2G), and results showed as the hydrodynamic diameter of the particles gradually increase after 3 days, most probably because of protein corona formation, as suggested by the literature [25].

Overall, we can conclude that neither the surface functionalization or the drug loading significantly changed size, ζ-potential, or stability of nanoparticles.

Concerning drug loading and release, in a previous study, we reported that nutlin-3a loaded in ApoE-PNPs was found to be 2.5 ± 0.7 wt%, while the release of drug was 12.5 ± 0.3% at pH 4.5 after 48 h upon ultrasound stimulation [15].
3.2. Biocompatibility assessment

The cytocompatibility of the nanoparticles and of the free drug was assessed in vitro on human endothelial cells using WST-1 colorimetric assay. There is no significant effect on cell culture metabolism of hCMEC/D3 cells treated with 0.2–25 μM of free Nut (Fig. S3, p > 0.05), 100–1000 μg/mL of PNPs, 100–1000 μg/mL of Nut-PNPs, 100–1000 μg/mL of ApoE-PNPs, or 100–1000 μg/mL of ApoE-Nut-PNPs (Fig. 2A, p > 0.05), suggesting the biocompatibility and the safety of the nanoparticles. Following experiments were carried out by using a safe concentration of 500 μg/mL of nanoparticles, and the corresponding concentration of Nut 21.5 μM (for comparison with drug-loaded nanoparticles).

We tested hemolytic activity in order to preliminarily determine whether nanoparticles present any issue that could prevent their interaction with blood, and thus their safety for clinical translation. As depicted in the inlet of Fig. 2B, showing a representative photo of the hemolysis test, the supernatant of the positive control (C+) was completely red and transparent, demonstrating a complete disruption of RBC membranes. Conversely, supernatants following nanoparticle treatment suggest no significant hemolysis, being colorless like the negative control (C−). The quantitative assessment (Fig. 2B) confirmed no significant hemolytic phenomena, being the hemolysis percentage 0.8 ± 0.8%, 1.2 ± 0.4%, 0.4 ± 0.2%, and 0.4 ± 0.4% for PNPs, Nut-PNPs, ApoE-PNPs, and ApoE-Nut-PNPs, respectively (p > 0.05), suggesting the safety of the nanoparticles at the tested concentration.

3.3. Nanoparticle/cell interaction

Upon treatment with DiO-labelled Nut-PNPs or ApoE-Nut-PNPs, the cellular localization of the nanoparticles was qualitatively assessed by using confocal microscopy after 24 and 72 h of incubation (Fig. 3). Representative confocal microscopy images of fluorescently labelled nanoparticles in green, f-actin in red, and nuclei in blue are reported as 2D images and 3D rendering, showing nanoparticles associated with the hCMEC/D3 tubes at 24 h (Figs. 3A) and 72 h (Fig. 3B). Samples incubated with ApoE-Nut-PNPs showed a remarkable time-dependent increase of nanoparticles interfaced to tubes. In general, a higher nanoparticle signal can be observed in cultures treated with ApoE-Nut-PNPs with respect to those ones incubated with non-functionalized Nut-PNPs, at both time points, suggesting the effectiveness of the functionalization in promoting nanoparticle-cell interaction. The same trend can be observed for non-loaded PNPs and ApoE-PNPs (Fig. S4).

3.4. In vitro tube formation

Although angiogenesis is a complex process occurring during cancer progression, tube formation is considered one of its key steps. In vitro endothelial tube formation assay was carried out on hCMEC/D3 cells by
Fig. 3. Internalization of DiO-stained Nut-PNPs and ApoE-Nut-PNPs (nanoparticles in green, f-actin in red, nuclei in blue). Representative single z-stack confocal images (top) and 3D confocal rendering (bottom) of hCMEC/D3 cells at A) 24 h and B) 72 h of incubation. For 3D rendering, scan area size is $x = 212 \mu m$, $y = 212 \mu m$, $z = 110 \mu m$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

considering 8 experimental groups to investigate the potential inhibitory effects of particles on vessel formation in the presence or absence of US stimulation. Endothelial cells were mixed with the nanoparticles, cultured on Matrigel matrix for differentiation and formation of tube-like structures, and visualized using bright-field microscopy imaging (Fig. 4A). The qualitative assessment of the tube formation shows the stunted growth of the vessels in ApoE-Nut-PNPs + US stimulated cultures; however, the tubular vessels matured in all other experimental classes. The ability of hCMEC/D3 cells to form tubular vessels was also quantitatively assessed by calculating the number of junctions, the total branching length, and the total mesh area as shown in Fig. 4B, C, and 4D, respectively. In good agreement with bright-field microscopy images, the analysis of ApoE-Nut-PNPs + US stimulated cultures showed a significant reduction in their length, junctions, and mesh area. The number of junctions significantly decreased to $191 \pm 3$ (p < 0.05) in ApoE-Nut-PNPs + US stimulated cultures compared to control cultures (276 ± 25 for Control; 252 ± 23 for Control + US). A similar trend for ApoE-Nut-PNPs + US treated cells was observed in total branching: the length was significantly reduced to 12,077 ± 125 μm (p < 0.05) compared to the Control (14,653 ± 666 μm) and the Control + US (13,802 ± 780 μm) cultures. In addition, ApoE-Nut-PNPs + US stimulated cultures showed a significant decrease in total mesh area (231,463 ± 28,310 μm², p < 0.05), while the total mesh areas were 409,784 ± 43,501 μm² and 420,827 ± 27,252 μm² for Control and Control + US cultures, respectively. The results show the significant role of ApoE-Nut-PNPs in deforming the vessel formation in the presence of piezoelectric stimulation.

3.5. Transwell migration and invasion assays

In tumor angiogenesis, endothelial cell migration occurs towards the migratory cues that are released by the tumor cells: this considered, the migration and invasion ability of hCMEC/D3 cells were tested using transwell inserts for all the 8 previously mentioned experimental classes in the presence of the conditioned media obtained from T98G cancer cells. Fig. 5A depicts the representative images of migrated cells, showing a few cells migrated through the transwell insert upon ApoE-Nut-PNPs + US stimulation. In line with this qualitative observation, upon quantitative evaluation, a significant decrease in the number of migrated cells that crossed the Matrigel in the ApoE-Nut-PNPs + US stimulation was significantly decreased to 2441 ± 652 cells (p < 0.05) with respect to Control (10147 ± 463 cells), Nut (10578 ± 1480 cells), Apo-PNPs (10311 ± 536 cells), Apo-Eut-PNPs (9377 ± 474 cells), Control + US (8828 ± 1625 cells), and Nut + US (9814 ± 1289 cells), as reported in Fig. 5C. Additionally, a significant decrease in the number of cells undergoing invasion was found upon ApoE-PNPs + US treatment (3991 ± 483 cells, p < 0.05) with respect to control cultures.

3.6. 3D spheroid invasion assay

We further evaluated the inhibitory effect of Nut, ApoE-PNPs, and ApoE-Nut-PNPs on endothelial cells invasion capacity using the 3D spheroid invasion assay, where particles-treated hCMEC/D3 spheroids have been embedded in basement matrix including Matrigel. The representative images of spheroids for all the experimental conditions are shown in Fig. 6A. The images show that Control, Control + US, Nut, Nut + US, ApoE-PNPs, and ApoE-Nut-PNPs cultures break the extracellular matrix (ECM) confinement and invade out of the spheroids after 48 h; however, ApoE-PNPs + US and ApoE-Nut-PNPs + US cultures show a few cells undergoing invasion out of the spheroids, in agreement with the transwell insert invasion assay. The whole area of ApoE-PNPs + US and ApoE-Nut-PNPs + US treated spheroids significantly decreased to 138207 ± 38052 μm² and 130067 ± 31247 μm², respectively (p < 0.05 with respect to the other experimental classes), being instead the whole spheroid area 300683 ± 12834 μm² for Control and 249119 ± 45842 μm² for Control + US, as reported in Fig. 6B. These data confirm that ApoE-PNPs- and ApoE-Nut-PNPs-mediated piezoelectric stimulation inhibits cell invasion, an important step in tumor angiogenesis.

3.7. Investigation of angiogenesis-related cytokines

Secretion of angiogenesis-related cytokines has been evaluated for all the 8 considered experimental classes as shown in Fig. 7 and Fig. S5. A significant reduction (p < 0.05) was observed concerning IL-6 and FGFb in ApoE-Nut-PNPs + US treated cultures. The release of IL-6 was strongly decreased to 32.0 ± 0.1 pg/μg (p < 0.05) with respect to the controls (Control = 59.3 ± 4.4 pg/μg; Control + US = 59.3 ± 6.8 pg/μg), as well as in comparison to all the other 8 experimental conditions. Furthermore, IL-6 levels were significantly reduced to 39.7 ± 4.7 pg/μg in ApoE-PNPs + US treated spheroids in comparison to control cultures. Concerning FGFb levels, a significant reduction was observed just in the ApoE-Nut-PNPs + US treated cultures (61.5 ± 2.1 pg/μg) compared to the Control (100.7 ± 10.9 pg/μg). Concerning the other investigated cytokines (IGF-1, EGF, VEGF, TGFb, TNFa, and leptin), no significant differences were found among all the 8 experimental classes (Fig. S5).

4. Discussion

Cancer cells require a constant supply of nutrients and oxygen, together with the removal of waste materials, because of their high metabolic rate. In this context, angiogenesis plays a fundamental role in tumor progression; moreover, apart from serving as nutrient and waste...
transporter, it also facilitates the dissemination of cancer cells to distant sites, leading to metastasis [26]: inhibiting angiogenesis is thus a promising methodology to hinder cancer progression.

Among numerous types of therapeutic strategies (such as combining anti-angiogenic drugs with chemotherapy or immunotherapy), the nanotechnology-based approach has emerged as a new treatment strategy in tumor-associated angiogenesis [27]. Nanoparticles can be used as effective tools owing to several advantages over plain drugs, providing high payloads of the therapeutic agents along with reduced toxicity and increased half-life, besides selective targeting thanks to the easy tailoring of the particle surface. In addition, these features might be used for fine-tuning the pharmacological profile of the drugs [28].

Fig. 4. Tube formation assay. A) Representative images of endothelial cells on Matrigel-coated plate during in vitro tube formation assay (t = 48 h); quantification of B) the number of junctions, C) the total branching length, and D) the total mesh area (*p < 0.05).
In this context, the present study was designed to assess the anti-angiogenic properties of ApoE-Nut-PNPs upon US stimulation. The particles showed monodisperse distribution and negative \( \zeta \)-potential value, which is typically characterized by higher biocompatibility and a slower cell uptake rate compared to nanomaterials with positive \( \zeta \)-potential values \([29]\), while ApoE functionalization enhances the interaction with hCMEC/D3 cells \([30]\). ApoE binds to the low-density lipoprotein (LDL) receptors, which are overexpressed in brain endothelial cells \([31]\). ApoE coating was reported to enhance particle crossing through the BBB by transcytosis \([32]\), and indeed our previous study showed an increment (about 20%) of ApoE-coated PNPs crossing an in vitro BBB model, with respect to non-functionalized PNPs \([15]\). Another group also showed an about 2-fold increment of cellular uptake of ApoE-functionalized solid lipid particles, suggesting the BBB crossing via a transcellular pathway \([33]\). In the present work, an increased hCMEC/D3 tube targeting efficacy was found to be associated with ApoE functionalization, independent from Nut loading. These results are in line with the literature, where nanoparticle functionalization with ApoE is exploited to enhance the BBB targeting via LDL receptor recognition. The increased incubation time increases the probability of ApoE binding to the LDL receptor of the brain microvascular endothelial cells, and, consequently, enhances nanoparticle accumulation at tube level \([34]\).

In tumor angiogenesis, the endothelial cells from the already existing vasculature spread for the formation of new blood vessels toward the direction of the tumor \([35]\). To observe this phenomenon, we cultured hCMEC/D3 cells together with particles on Matrigel matrix to show the potential inhibitory effect of the particles on vessel formation. The result of in vitro tube formation assay shows that ApoE-Nut-PNPs + US...
treatment disrupts the capillary tube formation in vitro at a non-toxic concentration (500 μg/mL) by decreasing the number of junctions, the total branching length, and the total mesh area, features that are considered to be critical for angiogenesis process [36]. The ability of ApoE-Nut-PNPs to suppress capillary tube formation could not be ascribed to a generalized cytotoxic effect, since the tested concentration does not affect cell viability. The effects of nutlin-3a on inhibiting vessel formation appeared to be ascribable to the upregulation of the p53 pathway and to apoptosis induction in human umbilical vein endothelial cells (HUVECs) [37]. Another study also shows that nutlin-3a suppresses either the tube growth and decreases the number of capillary connections in HUVECs by upregulating the p53 target genes MDM2, cyclin-dependent kinase inhibitor 1A p21 (CDKN1A/p21), and growth/differentiation factor-15 (GDF-15), along by inhibiting cell cycle progression [38]. Additionally, Su et al. found that low-intensity pulsed US inhibits in vitro tube formation of human endothelial cells via p38 mitogen-activated protein kinase (MAPK)-mediated activation of the endoplasmic reticulum stress response [39]. Although Nut is known to inhibit the vessel formations, we did not indeed observe any effect of the plain drug in our study; conversely, the piezoelectric stimulation enhanced the chemotherapeutic efficacy of Nut, in line with the literature [40].

Endothelial cell migration is one of the key steps of angiogenesis [41]; here, we performed a transwell insert migration assay, where particles-treated hCMEC/D3 cells were grown on the top chamber of the transwell membrane and migrated against the migratory cues secreted by cancer cells (T98G glioblastoma multiforme [42]). During the 48 h co-culture period, a few ApoE-Nut-PNPs + US treated endothelial cells migrated through the bottom chamber with respect to the other experimental classes. The literature reports as nutlin-3 treatment significantly decreases the migration of HUVECs co-cultured with lymph node carcinoma of the prostate (LNCaP) cells through up-regulation of TNF-α, matrix metalloproteinase 9 (MMP9), and C-X-C motif chemokine ligand 10 (CXCL10) [43]. Considering physical cues, it is worth mentioning the
effects of low-frequency magnetic fields, that were found to reduce the number of migrated HUVECs by causing an increment of cells in the G2/M phase of the cell cycle [44].

Cancer cell migration is intertwined with the invasion process, where cells should adhere to the ECM, degrade the basement membrane matrix, and infiltrate the ECM through a 3D process [45]. Here, the invasion ability of particles-treated endothelial cells via Matrigel-coated transwell membrane was tested against the migratory cues released by cancer cells. The number of invaded cells in ApoE-Nut-PNPs + US and ApoE-PNPs + US treated cultures was significantly reduced with respect to the control cultures, although no significant differences were found in the transwell insert migration assay. This may be due to the different mechanisms underlying migration and invasion processes, where invasion refers to the ability of cells to travel through the ECM within a tissue or to infiltrate into neighboring tissues, while cell migration is the directed movement of cells in response to a chemical or mechanical cue [46,47]. It was reported that nutlin-3 effectively inhibited HUVEC invasion in an MCF-7 (breast cancer cell line)-HUVEC co-culture system by activating Ras homolog family member A (RhoA) under hypoxic conditions [48]. In another study, nutlin-3 leads to inhibition of invasion and migration in gemcitabine-resistant hepatocellular carcinoma cells, leading to an increase of the expression of E-cadherin and a downregulation of the expression of vimentin, Snail, and Slug [49]. Weitz et al. instead showed that low-intensity focused US initiates a calcium wave via siRNA-mediated downregulation of both inositol trisphosphate receptors and transient receptor potential channels in invasive prostate (PC-3 and DU-145) and bladder (T24/83) cancer cell lines, leading to invasion inhibition [50].

To further evaluate endothelial cell invasion, a 3D spheroid invasion assay was carried out using particles-treated hCMEC/D3 cells on Matrigel basement matrix in the presence of cancer cell migration cues. The results showed a fewer number of cells undergoing invasion out of the spheroids in ApoE-Nut-PNPs + US and ApoE-PNPs + US treated cultures, which is in line with the transwell invasion assay. Literature reports as high-frequency ultrasound stimulation elicits cytosolic calcium waves in invasive prostate (PC-3 and DU-145) and bladder (T24/83) cancer cell lines, leading to invasion inhibition [50].

We prepared nutlin-3a-loaded, ApoE-functionalyzed, and remotely controlled piezoelectric nanoparticles exploited to inhibit tumor-induced angiogenesis. Our results showed that ApoE-Nut-PNPs have an in vitro anti-angiogenic activity by inhibiting critical steps in angiogenesis, including vessel formation, endothelial cells migration and invasion, and angiogenesis-related cytokines production. Moreover, the prepared piezoelectric nanovectors enhance the therapeutic efficacy of free drug after US stimulation. Altogether, our study opens a new avenue of using piezoelectric nanoparticles as feasible therapeutics to inhibit angiogenesis, encouraging further investigations towards clinical practice.

### Data availability statement

The data that support the findings of this study are available from the authors upon reasonable request.

### Author contributions

Ozlem Şen: Conceptualization; Investigation; Methodology; Writing – original draft. Atilio Marino: Investigation; Methodology; Data curation; Formal analysis. Carlotta Pucci: Investigation; Methodology; Data curation; Formal analysis. Gianni Ciofani: Conceptualization; Funding acquisition; Project administration; Writing – review & editing.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary data

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