Supplementary Information

Electro-mechanical coupling directs endothelial activities through intracellular calcium ion deployment

Changhao Li, Peng Yu, Zhengao Wang, Cheng Long, Cairong Xiao, Jun Xing, Binbin Dong, Jinxia Zhai, Lei Zhou, Zhengnan Zhou, Yan Wang, Wenjun Zhu, Guoxin Tan, Chengyun Ning*, Yahong Zhou* and Chuanbin Mao*

This PDF file includes:

Experimental Section

Fabrication of confined electric field (CEF)

Substrates used to construct CEF were obtained based on the previous method.[1] Then the focused laser beam from an IPG solid-state laser (λ = 1064 nm, Handslaser, China) was used to induce phase transitions on surface. The laser parameters of power and laser scanning speed were set as 3 W and 100 mm/s, respectively. The intervals of the CEF set as 40 μm, 60 μm, 80 μm, 100 μm, 120 μm by using the CAD software (HL software, Handslaser, China) to manipulate laser canning pathways. To acquire different electric field intensity, the samples were subsequently polarized under an applied electric field of ~ 0.5 kV/mm or ~ 1.5 kV/mm at 120 °C for 20 min.

Finite element modeling (FEM) of CEF

To study the dependence among the CEF created by the microdomains, the phase composition of material and the remaining polarization of the polarization voltage applied to the material, we performed FEM simulations with commercially available software COMSOL (5.3a, Burlington, USA). Through using finite element modeling to establish an electrostatics field model, the microscale electric field corresponding to the surface was simulated (formula : $E = -\nabla V$, $\nabla \cdot D = \rho_V$, $D = \varepsilon_0\varepsilon_r E + Dr$, D: electric flux, ρ_V : charge density, E : electric field intensity, V : potential, ε_0 : vacuum dielectric constant, ε_r : relative dielectric constant of the medium, Dr : residual polarization). The CEF was simulated by different dielectric constants caused by phase composition of the microdomains, and different polarization intensities that were defined in the model.

CEF characterization

The morphology of the CEF was characterized using a scanning electron microscope (SEM, EVO18, Carl Zeiss, Germany). Scanning Kelvin probe microscopy (SKPM, multimode8, Bruker, Germany) was employed to analyze the surface potential difference at the micro-boarder of CEF. The phase transition of CEF was analyzed using the X-ray diffraction (XRD, D8 Advance, Bruker, Germany) spectra. The P–E hysteresis loops was measured using an aixACCT TF Analyzer 2000 (aixACCT Systems GmbH, Dennewartstrasse, Germany). The relative permittivity was characterized by the impedance analyzer (4990A, Agilent, USA) from 10^2 Hz to 10^6 Hz at room temperature.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell (8000, USA), and

before passage 7 were used for the in vitro assay. The cells were cultured under standard conditions (37 ºC and 5% CO_2) in endothelial cell medium (ECM, ScienCell, USA) supplemented with 5% fetal bovine serum (FBS, ScienCell, USA), 1% endothelial cell growth supplement (ECGS, ScienCell, USA) and 1% penicillin/streptomycin solution (P/S, ScienCell, USA).

Cell proliferation assay

The Cell Counting KIT-8 (CCK-8, Dojindo, Japan) assay was used to measure the proliferation and viability of HUVECs seeded on samples placed in 48-well plates at a density of 1×10^4 cells/well, and cultured according to the above description. After the cells were cultured for 1, 4 and 7 days, cell proliferation was assessed using the CCK-8 assay. At each time point, the medium in each well was removed and the samples were rinsed with Phosphate Buffer Solution (PBS, pH = 7.4, Hyclone, USA). Then 200 μl of the ECM (which supplemented with 5% FBS, 1% ECGS and 1% P/S) and 20 μL of the CCK-8 solution were added to each sample and incubated at 37 °C for 2 h. The optical density (OD) value was measured at the wavelength of 450 nm by a microplate reader (Cytation 5, BioTek, USA).

Cell morphology and alignment characterization

F-actin cytoskeletal staining was conducted to examine the cell morphology and alignment. HUVECs seeded on samples placed in 48-well plates at a density of 1×10^4 cells / well, and cultured according to the above description. After cultured for 48 h, the samples were fixed with 4% paraformaldehyde (Biosharp, China) for 10 min. After washed with PBS containing 0.1% Triton X-100 (Sigma Aldrich, USA), the samples were sequentially stained with an Actin-Tracker Green (1:200 in PBS solution, 1 h, Beyotime, China) solution and DAPI (1:1000, 5 min, Beyotime, China) solution for F-actin cytoskeleton and cell nucleus visualization respectively. Then the stained samples were observed by a confocal laser scanning microscope (CLSM, TCS SP8, Leica, Germany). Fluorescence images were analyzed with ImageJ software (version1.42, NIH, Bethesda, USA) to determine two different parameters: circularity and aspect ratio. For each image, at least twenty cells from more than three fields were counted manually with the software used for cell morphology analysis. The cell circularity is defined as $(4\pi \times \text{cell area})/(\text{cell perimeter}^2)$: a circularity value of 1.0 indicates a circular shape, while decreasing values towards 0 indicate an increasingly elongated ellipse. The cell aspect ratio is defined as the ratio of the length of the Major Axis to the Minor Axis of an ellipse fitted to the cell area, which gives information about the cell elongation.[2] To assess the orientation of cells in a confocal image, two-dimensional Fast Fourier Transform (2D FFT) analysis was performed using ImageJ software supported by an oval profile plug-in (authored by William O'Connnell). It has been applied

to quantify collagen fibers or cell alignment in scanning electron microscopy and confocal images, as described previously.[3] After cropping and converting a color image into a grayscale 8-bit image with 1024 × 1024 pixels, the image is subjected to FFT analysis using ImageJ to obtain an output image containing white pixels. By placing a circular projection on the FFT output image and radial summing the pixel intensities for each degree between 0 degrees and 180 degrees, a graphical depiction of the FFT frequency distribution can be generated. Because the FFT frequency distribution is symmetric, the final statistical pixel intensity is between 0 and 180°. If the cells were randomly oriented, the peak intensity in the frequency chart would be at a random angle.

Transwell endothelial cell migration assay

To examine the effect of CEF on endothelial cell transmigration, a transwell migration assay was performed using 24-well transwell chambers (Corning, USA) with 6.5-mm-diameter polycarbonate flters (8-μm pore size). The samples were placed in the lower chamber of a 24-well transwell plate and fixed with an appropriate amount of agarose using a Pasteur pipette. Then 100 ul of HUVECs resuspension with a density of 1×10^5 cells were loaded into the upper chamber of the transwell plate, and 600 μL of ECM containing 5% FBS was added to the lower chamber. After 10 hours of incubation, the cells on the surface of the upper chamber were lightly wiped off using a cotton swab. The cells that migrated to the surface of the lower chamber were fixed with 4% paraformaldehyde for 10 min, then stained with 0.5% crystal violet (Beyotime, China) for 20 minutes, and observed by an inverted fluorescence microscope (Eclipsc Ti-U, Nikon, Japan), and finally at last three regions were photographed and counted.

Tube formation assay

To evaluate the role of CEF in promoting angiogenesis, an in vitro angiogenesis assay was performed using ECMatrix™ (Corning, USA). The tube forming assay was performed by placing the Matrigel on the surface of the material. The samples were put in a 24-well plate and the ECMatrix™ was covered on the surface according to the manufacturer's instructions. Then, the samples were incubated at 37 ℃ for 2 h to allow solidification of the matrix solution. HUVECs ($1*10^5$ cells per well) were inoculated with 1 ml of the ECM with 1% FBS and 1% ECGS and cultured at 37 ℃ and 5% CO_2. After cultured for 12 h, the samples were washed three times with Dulbecco's Phosphate Buffered Saline (DPBS, calcium/magnesium free, Gibco, USA) and subsequently stained with a Calcein AM (1 mg/ml in DPBS, 15 minutes, Aladdin, China) solution for marking live cells and emitting strong green fluorescence under CLSM. The cells were photographed from at least five random microscopic fields

using CLSM. The capillary tube branches number and tube length were analyzed with ImageJ software to quantify the angiogenesis process.

Chick chorioallantoic membrane (CAM) assay

The CAM assay was based on the procedure described used in many previous studies.[4] Pathogen-free fertilized E5 chicken embryos were purchased from Poultry Center of South China Agricultural University and preincubated at 37 °C and 60% humidity for 1 day. A hole was created at the pointed end of the egg and the vascular zone was identified on the CAM. Additionally, a 1.5 × 1.5 cm window in the shell was sectioned to expose the CAM. Then the samples were sanded to a thickness of 0.4 mm and a diameter of 6 mm, which were placed upside down on the CAM. A few drops of PBS were added in advance to keep the environment around the samples moist. After 4 days on the CAM, bright field images of the scaffolds and surrounding vasculature resulting from the angiogenic response were captured using a Canon single-lens reflex camera (EOS 800D, Canon, Japan) shooting in macro mode. To quantify angiogenesis, the total junction, tubule length and size were analyzed with Angioquant software (version1.33, Mathwork Inc.).

Quantitative real-time polymerase chain reaction (qRT-PCR)

The expression of genes associated with angiogenesis was assessed by qRT-PCR. HUVECs seeded on samples placed in 48-well plates at a density of 1×10^4 cells / well, and cultured according to the above description. After the cells were cultured for 4 and 7 d, the medium in each well was removed and the samples were rinsed with PBS. Then total RNA was extracted from samples for each kind of surface using HiPure Total RNA Kits (Magentec, China) and reverse transcribed into cDNA using a PrimeScript® RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology, Japan) according to the manufacturer's protocol. The RNA concentration was quantified by using a NanoDrop2000 spectrophotometer (Thermo Scientific, USA). RT-PCR reactions were performed using an SYBR Green System (Invitrogen, USA). Samples were held at 95 °C for 30 s, followed by 34 cycles at 95 °C for 5 s and 60 °C for 30 s. Primer sequences used were as follows. GAPDH (forward: 5'-GAAGGTGAAGGTCGGAGT-3'; reverse: 5'-GAAGATGGTGATGGGATTTC-3'), VEGF-A (forward: 5'-TACCTCCACCATGCCAAGTG-3'; reverse: 5'-ATGATTCTGCCCTCCTCCTTC-3') and eNOS (forward: 5'-CGGCATCACCAGGAAGAAGA-3'; reverse: 5'-GCCATCACCGTGCCCAT-3'). GAPDH was used as the housekeeping gene to normalize the results. The ΔΔCt-value method was used to calculate the relative expression values. All samples were analyzed in three biological replicates.

Immunofluorescence analysis

Immunofluorescent staining of VEGF-A was used for examining the expression of factors associated with angiogenesis. HUVECs were seeded on the CEFs and controls, placed in 48-well plates at a density of 5×10^3 cells / well culturing for 7 days. The samples were rinsed with PBS for three times and fixed with 4% paraformaldehyde for 15 min. After washed for three times, the fixed cells were penetrated with 0.1% Triton X-100 for 15 min. The 1% PBS solution of bovine serum albumin (BSA, Sigma Aldrich, USA) was used to block the samples. After the block solution was removed, the anti-VEGFA antibody (Abcam, USA) diluted at a ratio of 1:100 was added to the samples surface, respectively, and the mixtures were incubated at 37 °C for 1 h in dark. Then the samples were cultivated with goat anti-mouse IgG antibody (Servicebio, China) for 30 min. The immunostained samples were observed by CLSM. Further quantitative analysis of the fluorescent images of the stained proteins was conducted using the ImageJ software.

Measurement of intracellular calcium ions

The concentration of intracellular Ca^{2+} was determined using a green fluorescent dye, Fluo-4AM (Beyotime, China). HUVECs were seeded on samples placed in 48-well plates at a density of 2×10^4 cells/well, and cultured according to the above description. After cultured for 48 h, the cells on the samples surface were washed with DPBS, and then incubated with 5 μmol/L Fluo–4AM in DPBS for 30 min at 37 °C in the dark. In addition, two methods were used to detect the effects of intracellular and extracellular entry of calcium ions on the experiment: (1) Activating/blocking the Piezo1 in the plasma membrane with 20 μM Yoda1(MCE, USA) or 2.5 μM GsMTx4 (MCE, USA) to detect dependence on $[Ca^{2+}]o$; (2) Blocking the release of calcium ions from the endoplasmic reticulum with 10 μM U73122 (MCE, USA) to detect the dependence on $[Ca^{2+}]i$ stores. After different treatments, the cells were observed using CLSM and further quantitative analysis was performed using the ImageJ software. Here, the relative $[Ca^{2+}]i$ fluorescence intensity was used to evaluate the $[Ca^{2+}]i$ level. For the control group, a cell was randomly divided into two halves to compare the relative fluorescence intensity of $[Ca^{2+}]i$, thus obtaining the $[Ca^{2+}]i$ distribution ratio. For the CEF group, the $[Ca^{2+}]i$ distribution ratio was obtained by comparing the relative fluorescence intensity of $[Ca^{2+}]i$ between the irradiated area and non-irradiated area of the same cell.

Membrane potential measurements

Membrane potential was assessed by fluorescence spectroscopy using the fluorescence dye DiBAC4(3), which is a lipophilic anionic fluorescent dye correlated with changing membrane

potential. The HUVECs in the culture flask were digested into a suspension, centrifuged and washed once with 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma Aldrich, USA) buffer. The cells were then added with 5 μmol/L of DiBAC4 (3) (Dojindo, Japan), which was diluted with 20 mmol/L HEPES buffer. The number of cells was finally diluted to 5 *10^4 / mL. The cells were then incubated at 5% CO_2, 37 ° C. After the cells were cultured for 30 minutes, 500 μL of the above cell suspension was added to each well of 48-well plates with samples attached to the bottom to ensure that the number of cells per well was kept at 2×10^4, and then 48-well plates were cultured at 5% CO_2 at 37 °C for 30 minutes. Subsequently 100 μL of solution was removed from each well and transferred to a 96-well plate. Fluorescence intensity changes were measured by a flow cytometry with a Guava® easyCyte 6HT-2L Benchtop Flow Cytometer (Millipore, America), and data were analyzed using NovoExpress software (version1.2.5, ACEA Bioscience, USA).

Piezo1 interference and western blot analysis

HUVECs at 80~90% confluence were transfected with 50 nM siRNA using Lipofectamine 2000 in serum-free ECM according to the manufacturer's instructions (Invitrogen, USA). Sequences of siRNA probes (GenScript, China) were given as follows. Control (Scrambled): UUCUCCGAACGUGUCACGUdTdT; Piezo1 KO: AGAAGAAGAUCGUCAAGUAdTdT. Fresh ECM was replaced after 6 h and the cells were analyzed 72 h after transfection. Cell lysate was obtained by adding lysis buffer, and a BCA protein assay kit (Thermo Fisher, USA) was used for quantitative measurements. Then 30 μg of protein lysate (determined by Bradford assay) were loaded on 6% polyacrylamide gels at 150 V for 1 h, and transferred to polyvinylidenefuloride (PVDF) membranes, followed by addition of 5% skimmed milk to block for 1 h. Next, the membranes were incubated with the primary antibodies Anti-PIEZO1 (Abcam, USA) or Anti-GAPDH (AmyJet, China) at 4 °C overnight. Another day, the corresponding secondary antibody was used to incubate for 2 h. Immunoblots were visualized by using an enhanced chemiluminescence kit (Amersham, UK). The density of each protein band was calculated using ImageJ software.

Whole-cell patch-clamp recording

Electrical signals were amplified and recorded using an MultiClamp 700B amplifier and pCLAMP 10 software (Molecular Devices, USA) for whole-cell recordings of HUVEC. Pipette resistances after fire-polishing and filling with pipette solution were 3 - 5 MΩ. Recordings were made at room temperature with a bath solution containing: 130 mM NaCl, 5 mM KCl, 8 mM D-glucose, 10 mM HEPES, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, titrated to pH 7.4 with NaOH.[5] The pipettes were filled with

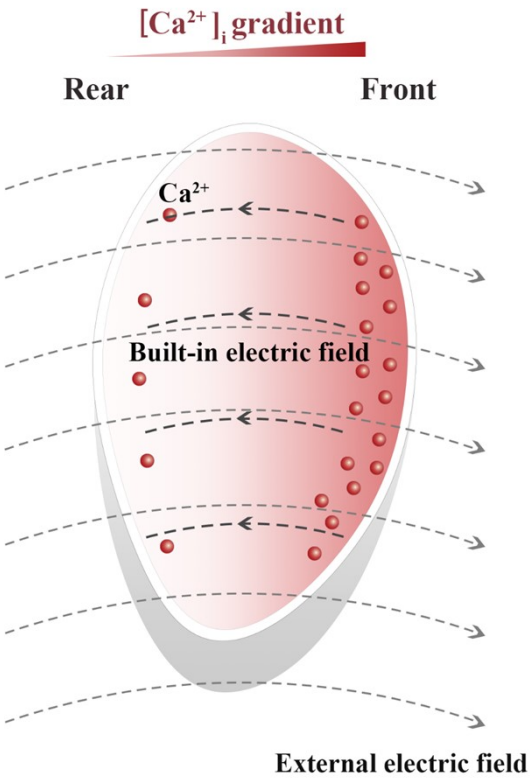
an internal solution containing: 95 mM Na-aspartate, 40 mM CsCl, 10 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM EGTA, 10 mM TEACl, titrated to pH 7.2 with CsOH. Correction was made for a calculated - 9 mV liquid-liquid junction potential. Data were filtered during acquisition with a low-pass filter of 2 kHz, and analyzed offline with Clampfit 10.2 (Molecular Devices, USA).

Intracellular nitric oxide (NO) measurement

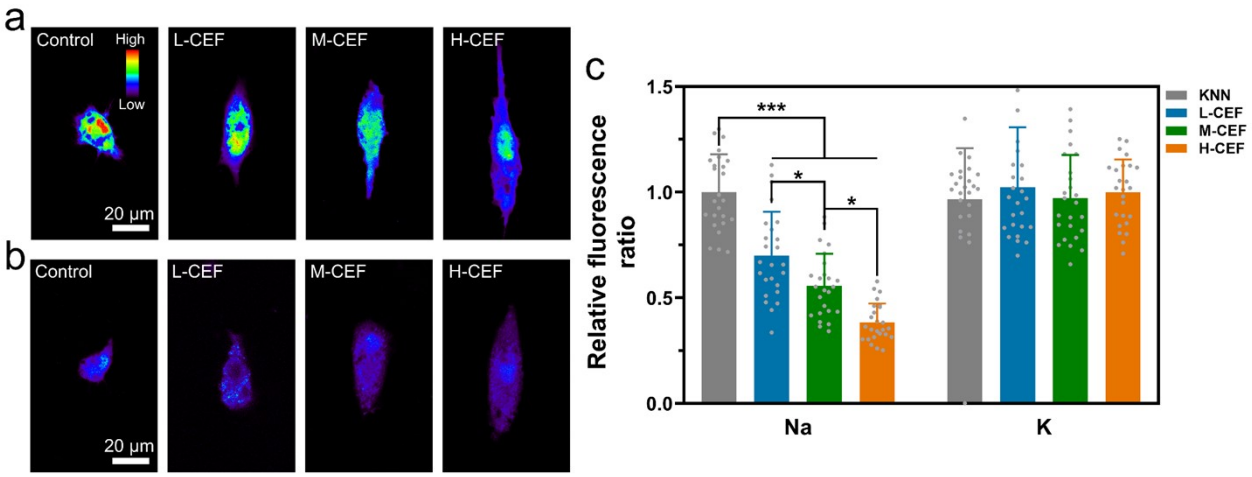
To evaluate the level of intracellular NO, the fluorescent probe 3-amino-4-(aminomethyl)-2′,7′-difluorescein, diacetate (DAF-FM DA, Beyotime, China) was used. HUVECs were seeded on the samples placed in 48-well plates at a density of 2×10^4 cells/well, and cultured according to the above description. After cultured for 48 h, the cells on the samples surface were washed with PBS, and then incubated with 5 μmol/L DAF-FM DA for 30 min at 37 °C in the dark. The cells were observed using CLSM and further quantitative analysis of fluorescent images was done using the ImageJ software.

Statistical analyses

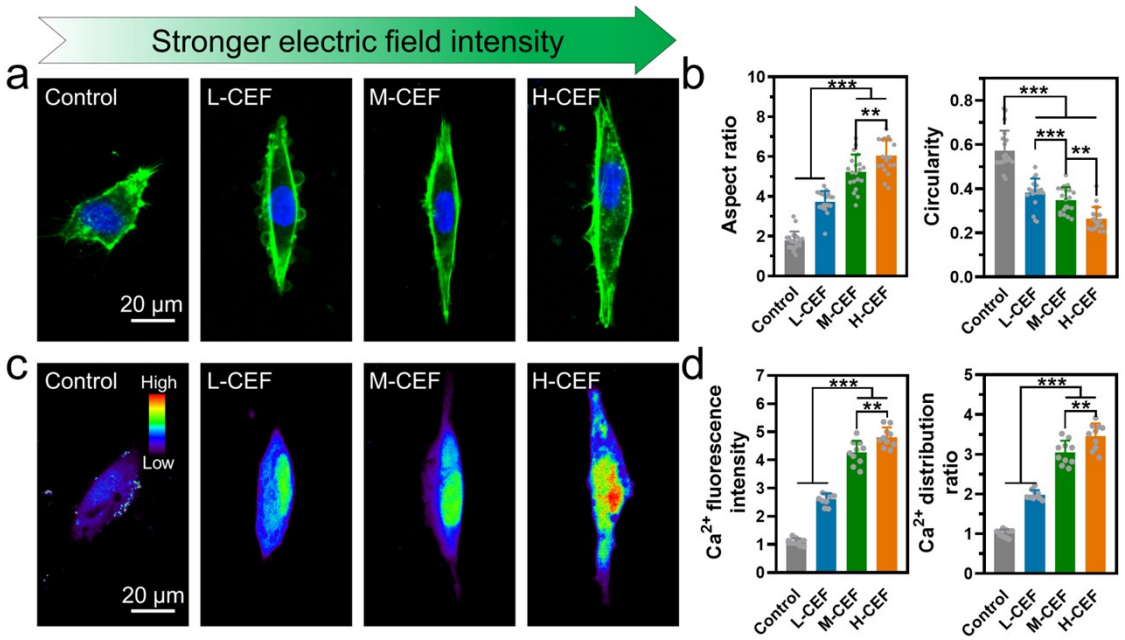
Data were presented as mean ± standard deviation (SD). Student's t test and one-way ANOVA with a multiple comparisons test were carried out, and a value of $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$ was considered statistically significant. All statistical analysis and data processing were performed using GraphPad Prism 8.0.1.



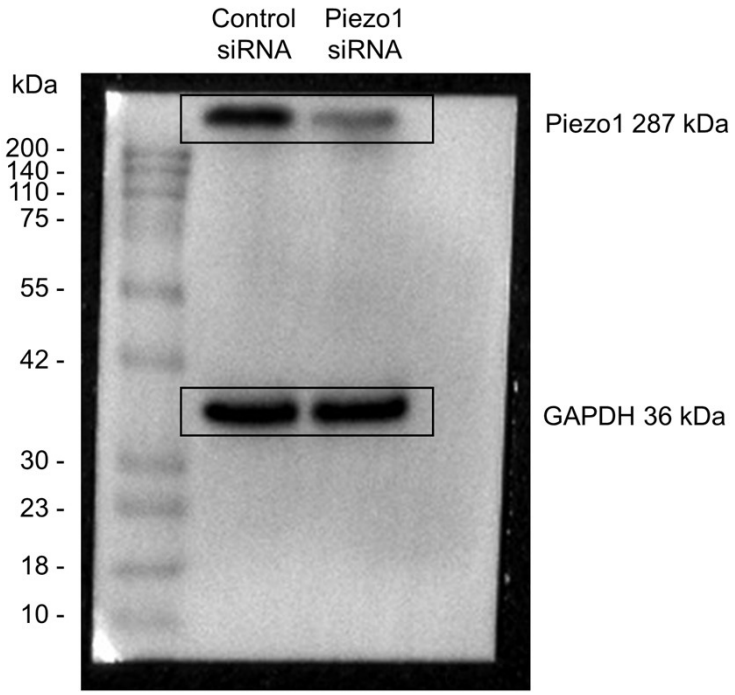
Supplementary Fig. S1 Schematic representation of the rear-to-front gradient along the direction of the external electric field vector generated by the polarization of $[Ca^{2+}]_i$). The vector direction of the external spatial electric field points from the high-potential (non-irradiated) region to the low-potential (laser-irradiated) region, and we define the cytoskeleton in the high-potential region and the low-potential region as the rear edge and the front edge, respectively.



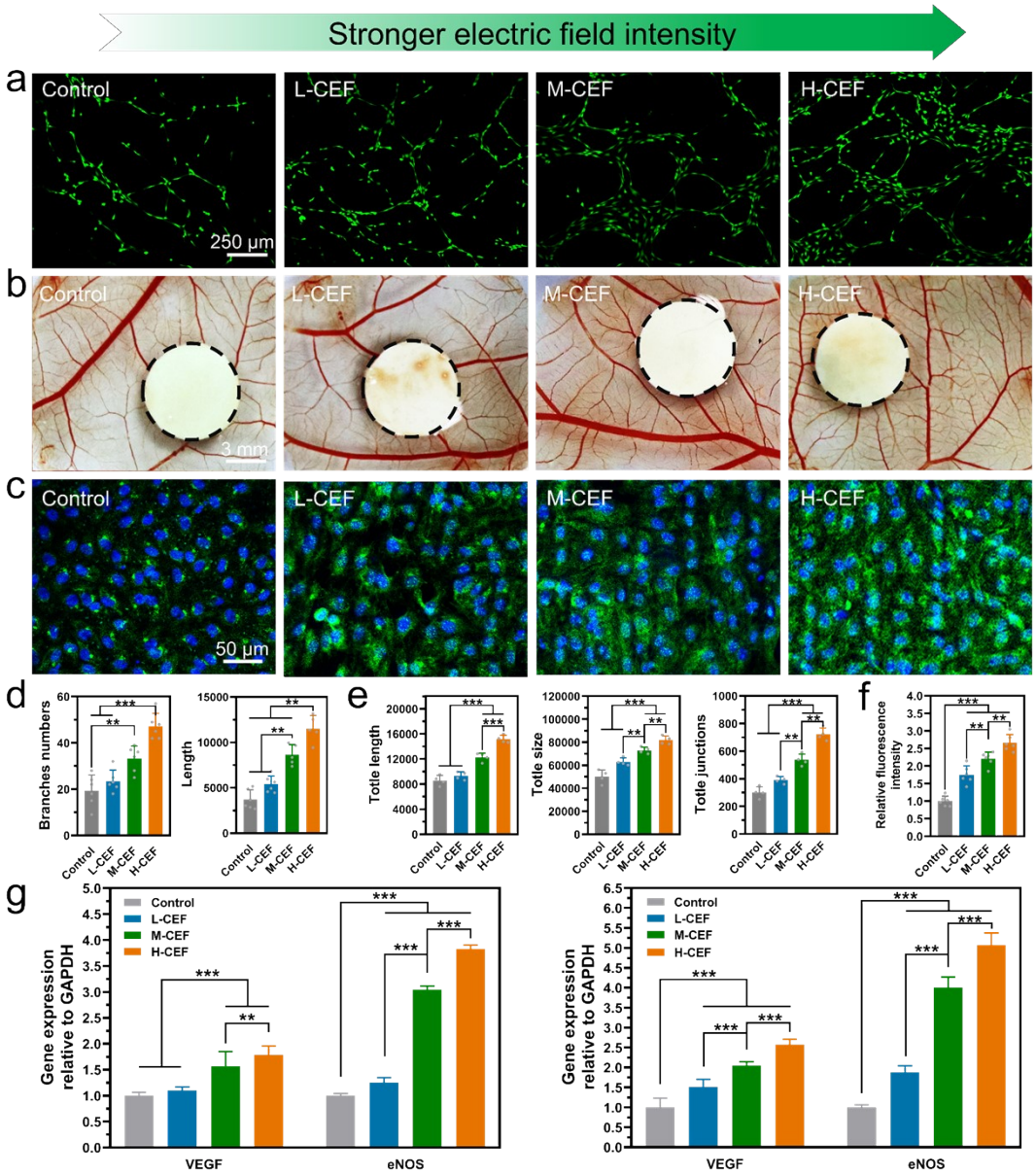
Supplementary Fig. S2 The staining of intracellular ions under CEFs with different intensities. (a) Potassium ions. (b) Sodium ions. (c) Quantitative analysis of relative fluorescence ratio by ImageJ software ($n = 25$). *$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$.



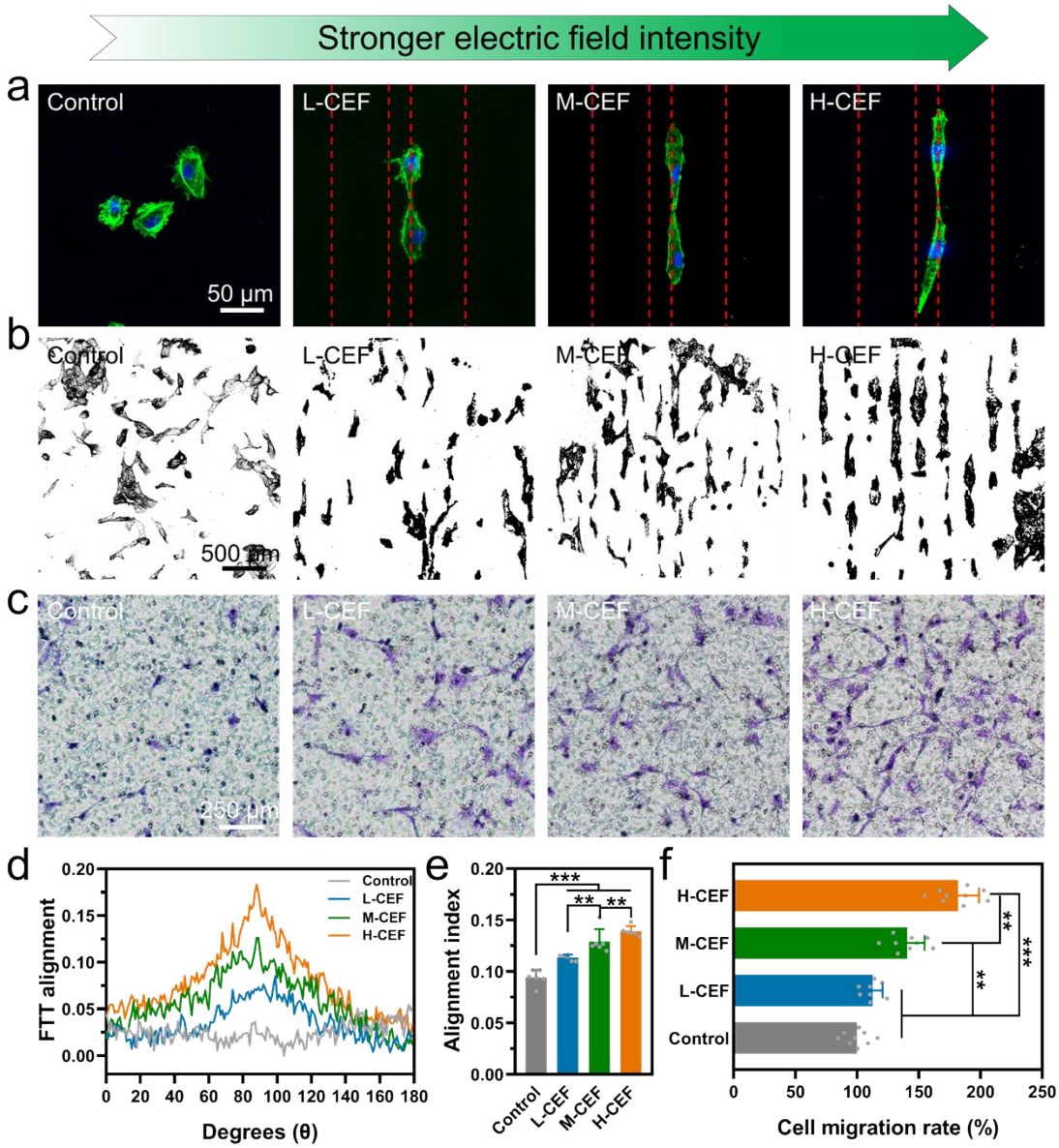
Supplementary Fig. S3 Calcium polarization and cell deformation under CEFs with different intensities. (a, c) Representative fluorescent images of cytoskeleton (a) and $[Ca^{2+}]_i$ (c) of HUVECs cultured for 48 h with a different range of CEF intensities. Pseudo-color is used for better identification of concentration gradients. (b, d) Quantitative analysis of cytoskeleton (b) (n = 20) and $[Ca^{2+}]_i$ (d) (n = 10) parameters by ImageJ software. *P < 0.05, **P < 0.01 or ***P < 0.001.



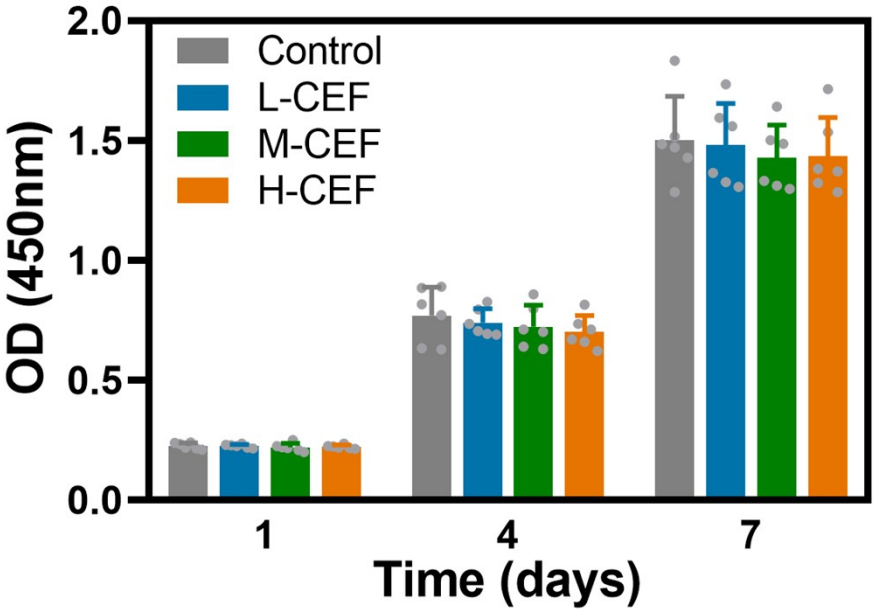
Supplementary Fig. S4 Full uncropped image of Western blot from Fig. 6a.



Supplementary Fig. S5 Performance of different CEFs in regulating angiogenesis. (a) Representative fluorescence image of tube formation assay in different sample groups. (b) Representative optical images of CAM in different sample groups. (c) Immunofluorescence staining of VEGF-A expressed by HUVECs cultured with different sample groups for 7 days. The results indicated that the CEF promote the expression of VEGF-A compared to control. The VEGF-A was stained in green color with FITC. The nuclear stain is in dark blue with DAPI. (d) Quantitative analysis of tube branches number, length of HUVECs (n = 4). (e) Quantitative analysis of relative fluorescence intensity based on VEGF-A staining images (n = 6). (f) Quantitative analysis of vessel length, size and junction of CAM by Angioquant software (n = 4). (g) Expressions of the angiogenesis-related genes in HUVECs cultured on CEFs and controls for 4 days (left) and 7 days (right) (n = 3). The results showed that CEF promote the expression of related angiogenic pathway growth factors. *P < 0.05, **P < 0.01 or ***P < 0.001.



Supplementary Fig. S6 Performance of different CEFs in regulating cell adhesion and spreading. (a) Cytoskeletal staining of HUVECs cultured on different sample groups for 48 h. Stripe interval is 80 μm, laser scanning width is about 25 μm. (b) Representative bright field pictures of FFT. (c) Representative bright field pictures of HUVECs migration using transwell membranes. (d) Pixel intensity plot of cell alignment was quantified by two-dimensional FFT analysis. (e) Comparison of "Alignment index" among various CEF (n = 6). CEF can induce the orderly arrangement of cells in the same direction. (f) The cell migration rate as a comparison method used to assess HUVECs migration (n = 9). *P < 0.05, **P < 0.01 or ***P < 0.001.



Supplementary Fig. S7 The cell viability of HUVECs cultured on different CEFs for 1, 4, and 7 days was measured by CCK-8 assay. It indicated that CEF have good cell viability and proliferation ability (n = 6).

References

1 C. Li, Y. Li, T. Yao, L. Zhou, C. Xiao, Z. Wang, J. Zhai, J. Xing, J. Chen and G. Tan, *ACS Appl. Mater. Interfaces*, 2020, **12**, 34505-34513.

2 X. Q. Liu, L. Fourel, F. Dalonneau, R. Sadir, S. Leal, H. Lortat-Jacob, M. Weidenhaupt, C. Albiges-Rizo and C. Picart, *Biomater.*, 2017, **127**, 61-74.

3 N. F. Huang, J. Okogbaa, J. C. Lee, A. Jha, T. S. Zaitseva, M. V. Paukshto, J. S. Sun, N. Punjya, G. G. Fuller and J. P. Cooke, *Biomater.*, 2013, **34**, 4038-4047.

4 Z. Chen, Z. Wen and X. Bai, *Bio-protoc.*, 2013, **3**, e913-e913.

5 J. Li, B. Hou, S. Tumova, K. Muraki, A. Bruns, M. J. Ludlow, A. Sedo, A. J. Hyman, L. McKeown and R. S. Young, *Nature*, 2014, **515**, 279-282.