Supplementary Information

Materials derived from the Human Elastin-like Polypeptide fusion with an antimicrobial peptide strongly promote cell adhesion

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HhBD1 cloning, expression, extraction, and purification

Cloning. The coding sequence of hBD1, flanked by *DraIII and HindIII* sites, was purchased from Eurofins Genomics (Milan, Italy) and ligated into the pEX8EL vector, exploiting the unique *DraIII and HindIII* sites in the vector for the in-frame fusion if the coding sequence of hBD1nat the C-terminus of HELP according to the already described methodology [1]. Chemically competent *Escherichia coli* C3037I cells (New England Biolabs, Massachusetts, USA) were transformed with the ligation mixture, and positive clones were selected and verified by sequencing (Eurofins Genomics).

Expression. Positive clones of *E. coli* C3037I strain transformed with the plasmid carrying the HhBD1 construct were grown in Luria-Bertani medium (LB, 10 g L^{-1} tryptone, 5 g L^{-1} sodium chloride and 5 g L^{-1} yeast extract, pH 7.2) supplemented with 50 µg mL^{-1} of ampicillin and 70 µg mL^{-1} of chloramphenicol. A starter culture of 120 mL in the same medium after overnight growth at 37 °C was used to inoculate 1.2 L of Terrific Broth (TB, 12 g L-1 tryptone and 24 g L-1 yeast extract) supplemented with phosphate-buffered glycerol (PGB, 2.3 g L^{-1} monobasic potassium phosphate, 12.5 g L^{-1} dibasic potassium phosphate, and 4 mL L^{-1} monobasic glycerol). Bacterial cells were grown at 37 °C under shaking conditions until turbidity at 600 nm reached about 1 OD unit. HhBD1 expression was then induced by adding Isopropyl β-d-1-thio galacto pyranoside to a final concentration of 0.1 mM, and the bacteria were further cultured for 5 hours. Then, the bacterial mass was harvested by centrifugation at 8000 rpm for 20 minutes at 10 °C (Beckman-Coulter, J-26 XP, California, USA), and the pellets were stored at -20 °C for further processing.

Extraction. The pellets obtained from the expression cultures were resuspended in 400 mL of extraction buffer (50 mM Tris/HCl pH 8, 250 mM NaCl, 0.1 mM EDTA, 0,1% Triton X-100, and 1 mM PMSF) and disrupted using a high-pressure homogeniser (Panda NS1001L, GEA Niro Soavi, Parma, Italy) by 4 cycles at 1280 bar. Then, 2-mercaptoethanol was added to a final concentration of 20 mM, the bacterial lysate was cooled on ice and centrifuged at 10000 rpm for 30 minutes at 8 °C (Beckman–Coulter, J-26 XP, California, USA), and the cellular debris was discarded, and the supernatant was stored at -20°C.

Purification. The HhBD1 purification procedure was based on the thermo-responsive properties of the HELP domain following a method known as Inverse Transition Cycling (ITC)[2]. Briefly, the supernatant from the extraction procedure described above was precipitated by adding NaCl to a final concentration of 1.5 M and warmed to 37 °C in a water bath. The aggregated polypeptide particles were collected by centrifugation at 7000 rpm at 37 °C for 30 minutes. The resulting pellet was redissolved in cold water; the insoluble material was removed after cold centrifugation. The supernatant was precipitated again by NaCl addition and raising temperature to 37 °C. Three successive ITC cycles ensured the production of highly pure recombinant fusion protein. After the last temperature-dependent transition cycle, the material was frozen and lyophilised for long-term storage.
A solution of 0.5 mg mL-1 concentration was prepared for SDS-PAGE analysis.

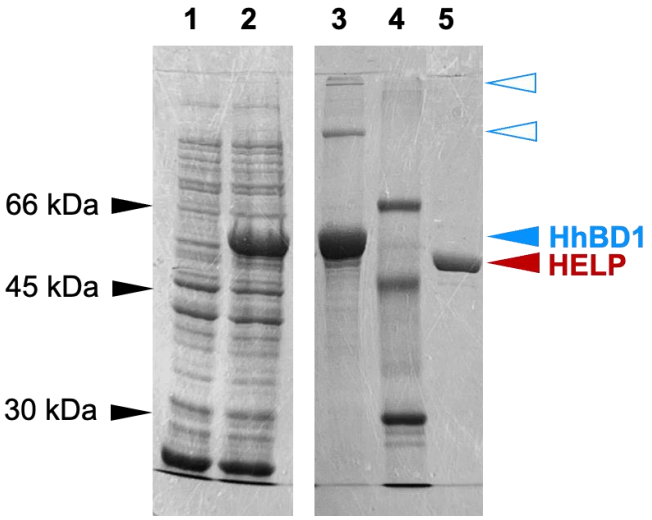

Figure S1: Representative 10 % SDS-PAGE analysis of the production and purification of the HhBD1 biopolymer. Lane 1, total protein content of the lysate of the expression culture before IPTG induction; lane 2, total protein content of the bacterial lysate 5 hours after IPTG induction; lane 3, recombinant HhBD1 fusion biopolymer purified by ITC. The main HhBD1 band (solid blue arrow) corresponded to an apparent mass of about 55 kDa. Minor, slower migrating bands are visible in the upper part of the lane, likely due to HhBD1 multimers formation (open blue arrows). Lane 4, molecular mass markers, and lane 5, purified HELP biopolymer as the reference. Molecular mass markers: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa. Coomassie blue staining.

Stability of HELP and HhBD1

Effect of pH. HELP and HhBD1 pH stability were tested by incubating 1mL of each biopolymer at the concentration of 2 mg mL^{-1} in 5 mM of HCl and 5 mM of NaOH. In parallel, aqueous solutions of the biopolymers were prepared as controls. The solutions were then incubated without stirring for 16 hours at 25°C.

Table S1. pH values of HhBD1 and HELP solutions in acid and basic conditions. The pH values of the solutions were measured at the beginning of the experiment (T_0) and after 16 hours of incubation (T_{end}).

	pH			
	HELP		HhBD1	
	T_0	T_{end}	T_0	T_{end}
H_2O	6.3	6.7	6.2	6.9
5 mM NaOH	12	11.9	12	11.9
5 mM HCl	2.3	2.3	2.3	2.2

At the same time points, 10 µL from each sample were collected and mixed (1:1) with Laemmli loading buffer for SDS-PAGE analysis. 4 µL (4 µg) were loaded per well to analyse the biopolymer stability using an SDS-PAGE.

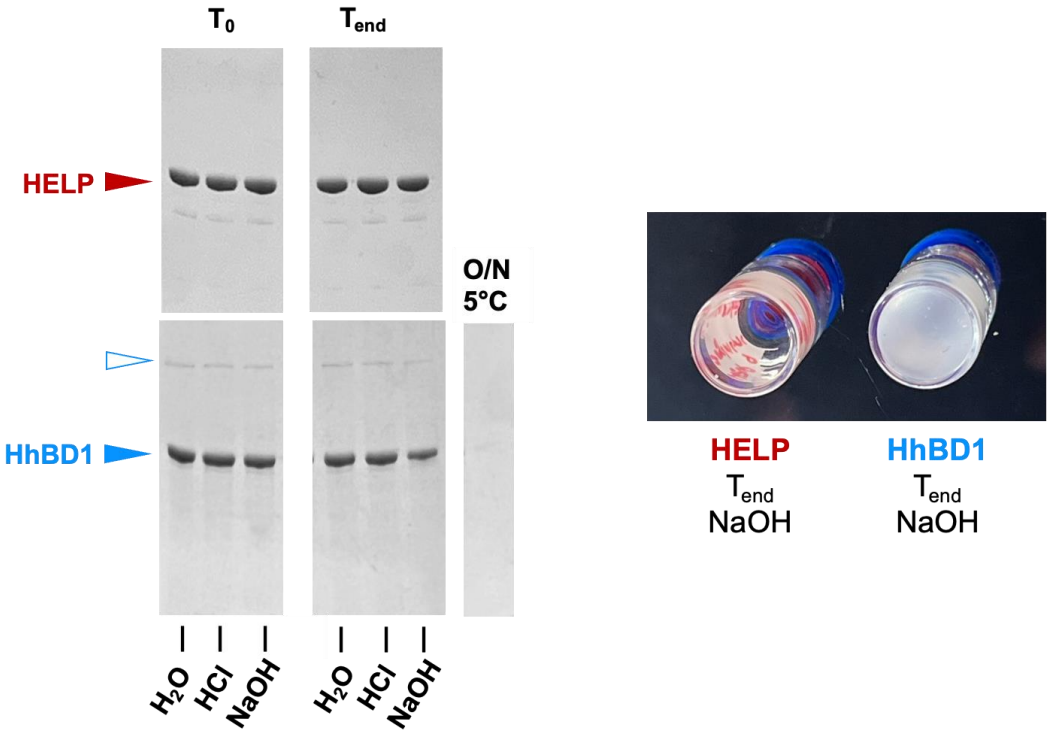

Figure S2: HELP and the HhBD1 biopolymers' stability in acidic and basic conditions. 9% SDS-PAGE analysis (on the left). After the run (V=160 v, 1 hour), the gel was stained with Coomassie blue. Red arrow, HELP electrophoretic band, solid blue arrow, HhBD1 electrophoretic band, open blue arrow, HhBD1 multimer. All the samples except the HhBD1 in 5 mM NaOH remained unaffected by the treatments. A concentration decrease was detectable by SDS PAGE analysis in the HhBD1 sample after incubation in basic conditions with the simultaneous formation of a clearly visible hydrogel-like layer on the tube bottom (on the right). This layer proved to be stable after cold water rinsing and subsequent overnight incubation at 5°C in 1 mL of water. No protein signal was detected by SDS-PAGE analysis of this sample.

Susceptibility to elastase. Aqueous solutions of HELP and HhBD1 biopolymers (6 mg mL^{-1}) in 10 mM NaPi buffer pH 6.8 were incubated with 0.2 (100X), 0.02 (10X) ng μL^{-1} of elastase (Sigma, #E7885) for 3 h at 37 °C in a final reaction volume of 50 µL. In parallel, control reactions were set up under the same condition without the elastase (CTRL). The same reactions were prepared with bovine serum albumin (BSA) in the absence and in the presence of the highest final concentration (0.2 ng μL^{-1}) of elastase. To stop the reactions, 50 µL of Laemli loading buffer were added to each sample, and 1.5 µL of this mixture, corresponding to 4.5 µg, were analysed in 9% SDS-PAGE. As shown in Figure S3, BSA was not degraded by the enzyme, whereas, as expected, HhBD1 showed the same elastase susceptibility of HELP, holding the potential for the smart release of the bioactive domain upon elastolytic stimuli.[3]

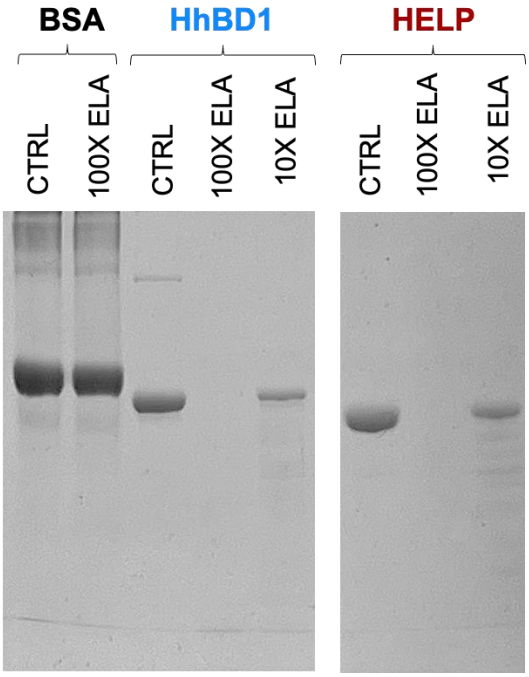

Figure S3: 9% SDS-PAGE analysis of HELP and HhBD1 biopolymers' susceptibility to elastase degradation.

Accessible surface area calculation of the 36 amino acids domain of hBD1

The PDB file of the 36 amino acids structure of hBD1 from PDB entry 1E4S (see **Fig. 1** in the main manuscript) was used to estimate the solvent-accessible surface area using the online software GETAREA (https://curie.utmb.edu/getarea.html).[4]

Table S2: Theoretical solvent accessibility surface area of hBD1

POLAR area/energy	=	962.47
APOLAR area/energy	=	1921.08
UNKNOW area/energy	=	0.00
Total area/energy	=	2883.55

Radial diffusion assays

Radial diffusion assays were performed as described in the main manuscript (**Experimental section 4.2**) in the absence or presence of 2 mM DTT.

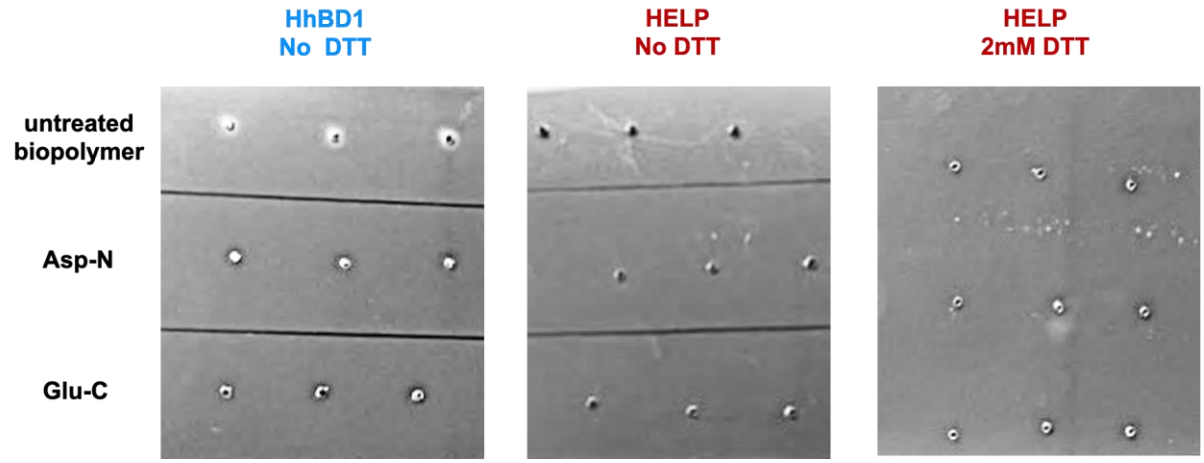

Figure S4: Representative images of the radial diffusion assays in the absence and presence of 2 mM DTT. No inhibition halos were observed.

Oscillatory rheology analysis

Frequency sweep analysis. Oscillatory rheology analysis was performed as described in the main manuscript (**Experimental section 4.3**) using a Malvern Kinexus Ultra Plus rheometer (Alfatest, Milan, Italy). The frequency sweep analysis was performed after the time sweep. The storage (G') and loss (G") moduli of the hydrogels were recorded from 0.1 to 10 Hz (stress 4 Pa, within the linear regime). For the graphical representation, the mean value of two representative data sets was plotted.

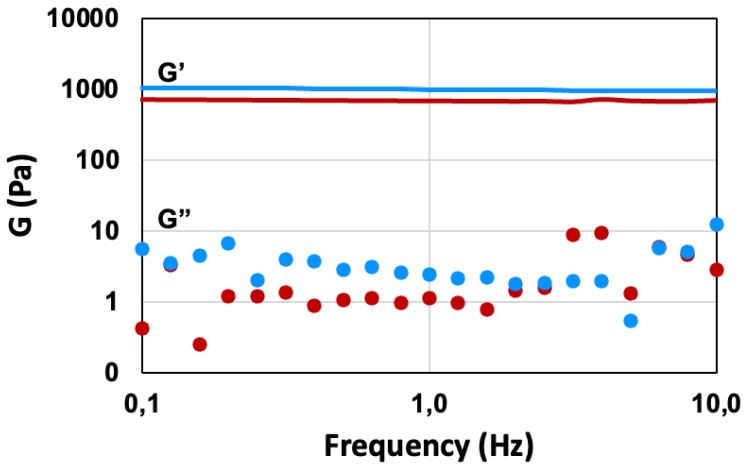

Figure S5: Frequency sweep analysis of HELP (red) and HhBD1 (blue) matrices. G' is the elastic or storage modulus and G" is the viscous or loss modulus.

Mass spectrometry

The hBD1 domains released from the matrix were analysed by electrospray ionisation mass spectrometry (ESI-MS). Specific cleavage of 4% (w/v) HhBD1 matrix was achieved by carrying out the reactions with Glu-C and Asp-N enzymes under the same conditions reported in the main manuscript (**Experimental section 4.3**). The matrices were prepared by depositing 10 µl of 4% HhBD1 aqueous solution per well in a 96-well polystyrene V-shaped bottom microplate (Sarstedt, Numbrecht, Germany). After extensive washing with water, the matrices were incubated with 25 µL of 100 mM ammonium bicarbonate buffer pH 8, containing 8.3 ng μL^{-1} or 2.6 ng μL^{-1} of Glu-C and Asp-N, respectively. In total, 8 replicates for each enzymatic reaction were set up. After the reaction, the supernatants of the matrices digested with Glu-C were pulled, as well as those of the matrices treated with Asp-N. For ESI-MS analysis, the pulled supernatants were supplemented with 50% acetonitrile/0.1% TFA and injected with a syringe infusion pump at a flow rate of 2 µl min^{-1}, with a scanning range of m/z 300/1800. Detection was carried out in positive ion mode with an orifice potential set at 75 V.

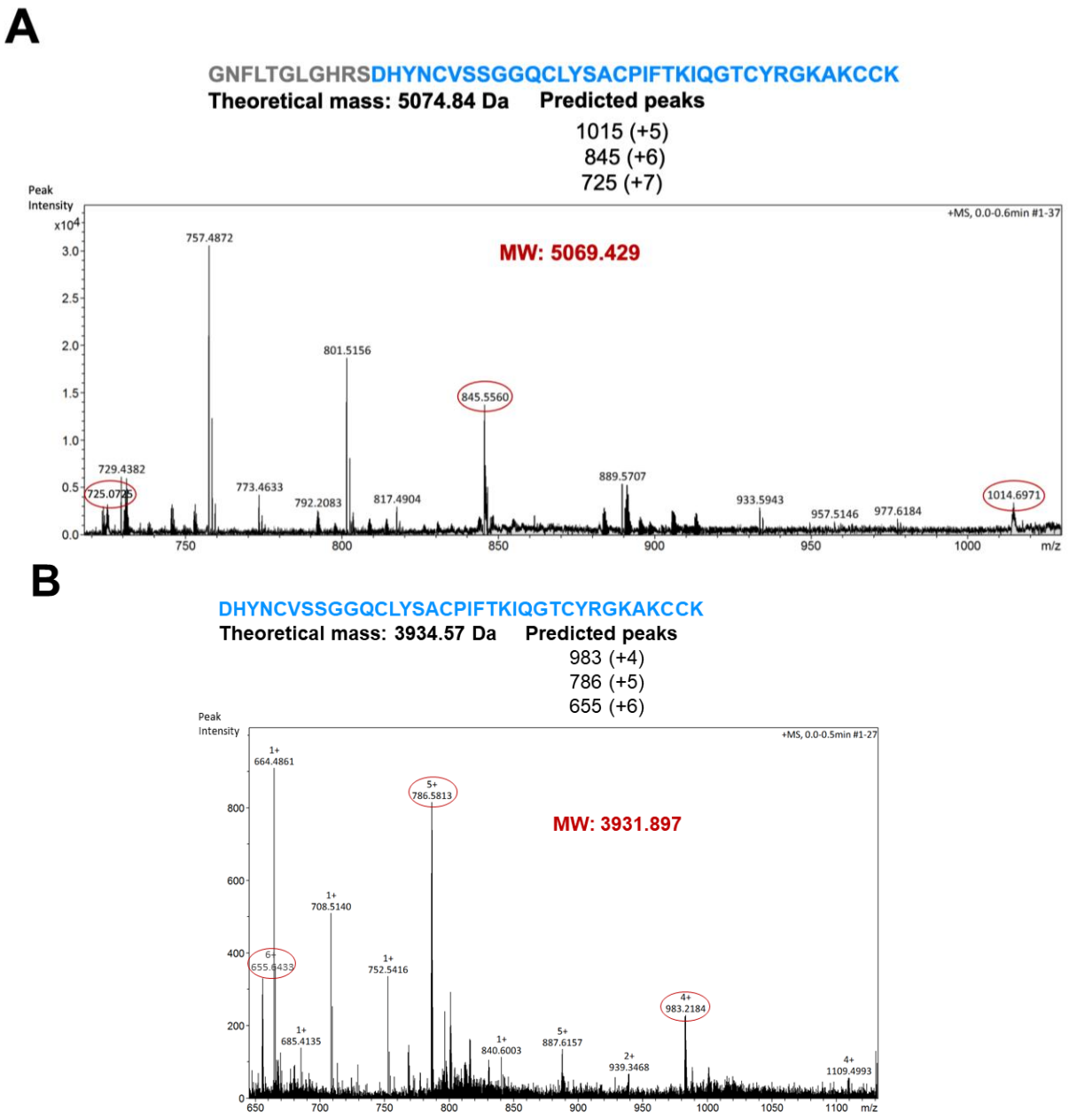

Figure S6: Mass spectrometry analysis of the peptides derived from the treatment of HhBD1 matrices with A) Glu-C and B) Asp-N endoproteinases.

Analysis of cells cultured on HELP and HhBD1 thin-film coatings

Fluorescence microscopy analysis was performed on cells seeded on the HhBD1 and HELP thin-films. Thin-film preparation, cell seeding, and fluorescence staining were performed as described in the main manuscript (**Experimental section 4.4**).

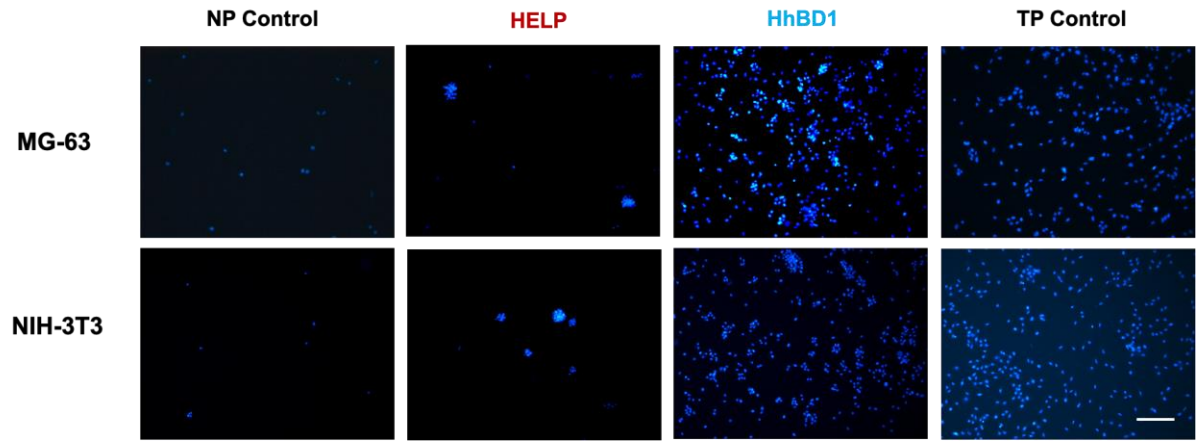

Figure S7: Fluorescence microscopy analysis of osteoblastic and fibroblastic cell adhesion on HELP and HhBD1 thin films (100 µg per 1 cm^2). Nuclei were stained with DAPI. The bar is 200 nm.

Analysis of cells seeded on HELP and HhBD1 matrices

Phase contrast and fluorescence microscopy analyses were performed on cells seeded on the HhBD1 and HELP matrices. Matrix preparation, cell seeding, and fluorescence staining were performed as described in the main manuscript (**Experimental section 4.4**).

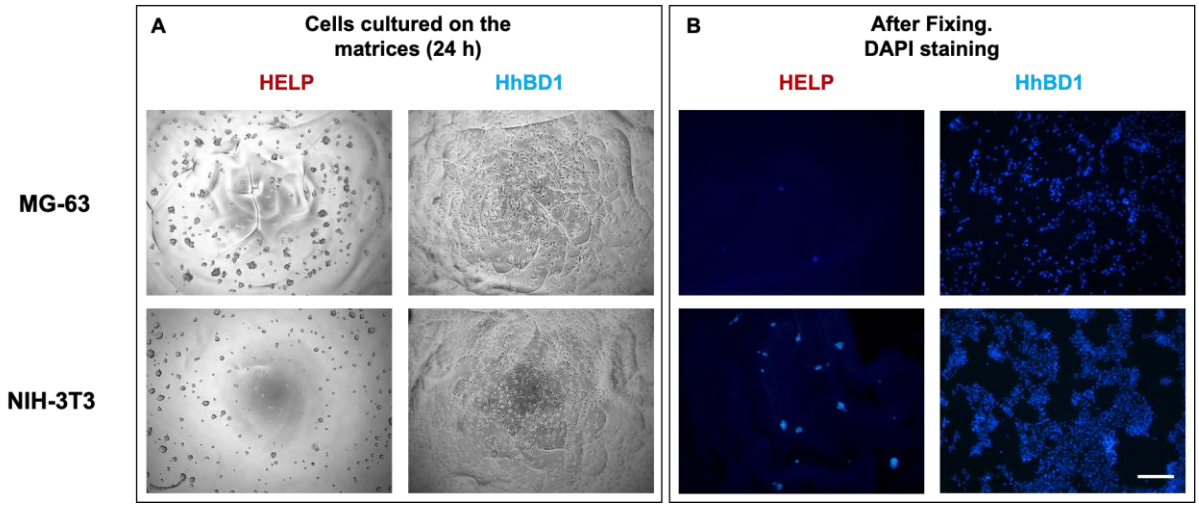

Figure S8: Fibroblast and osteoblast cell cultures on the HELP-based matrices. A) Representative contrast phase microscopy images of cell adhesion on HELP and HhBD1 matrices 24 hours after seeding. B) Fluorescence microscopy images of the nuclei of the cells attached to the matrices stained with DAPI. The bar is 200 nm.

References

1. A. Bandiera, A. Taglienti, F. Micali, B. Pani, M. Tamaro, V. Crescenzi and G. Manzini, *Biotechnol Appl Biochem*, 2005, **42**, 247-256.
2. D. E. Meyer and A. Chilkoti, *Nat Biotechnol*, 1999, **17**, 1112-1115.
3. A. Bandiera, A. Markulin, L. Corich, F. Vita, V. Borelli, *Biomacromolecules*, 2014, **15**, 416-422.
4. R. Fraczkiewicz and W. Braun *Journal of Computational Chemistry*, 1998, **19**, 319-333.