Supporting Information

# Synthetic Extracellular Matrices as a Toolbox to Tune Stem Cell Secretome

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## Experimental

*Polymer synthesis and peptide conjugation*

Polyisocyanides were synthesized as previously reported.[1] Briefly, polymers with different contour lengths were prepared by using a different monomer to catalyst ratio. For the short polymers **P1**, we used 1:1000; for the long polymers **P2**, 1:5000. All polymers contain 3.3% azide-appended monomers. Polymer lengths were measured by viscometry, with parameters determined previously:[2] $K = 1.4 \times 10^{-9}$ and $a = 1.75$. For biofunctionalization, the RGD peptide was equipped with a DBCO-terminated spacer. Next, the DBCO-$PEG_4$-GRGDS construct was reacted with the azide appended polymers following an previously described protocol,[1] such that on average 1% of the monomers in the polymer chain carry a peptide. The degree of RGD is controled by adding DBCO constructs to an excess of azide groups on the PIC co-polymer (1 eq. DBCO to react with 3.3 eq. $N_3$). The click chemistry reaction that we routinely employ (DBCO-N3 SPAAC reaction) has been selected because it is exceedingly efficienct with conversions of over 90% within 15 minutes, as we demonstrated in earlier work.[3] To ensure complete conversion, the conjugation reactions in this manuscript were conducted overnight. Due to the low concentrations on the PIC polymer, quantitative analysis is difficult and often not very precise.

*Cell culture*

hASCs were isolated from patients with informed consent and grown in α-MEM medium (Gibco, Thermo Fisher, USA) supplemented with 10% fetal bovine serum (FCS) (Gibco, Thermo Fisher, USA) and 1% Penicillin-Streptomycin (P/S) (stock concertation: 10,000 Units/mL Pen, 10,000 µg/mL Strep, Gibco, Thermo Fisher). Cells were used before passage 6.

Human fibroblasts were obtained from the RUMC cell repository bank and have been previously isolated from a biopsy of the pre-cervical region of the anterior vaginal wall of a

woman after a hysterectomy for benign gynecological reasons, as previously reported.[4] Fibroblasts were grown in low glucose DMEM medium (Gibco, Thermo Fisher) supplemented with 10% FCS (Gibco, Thermo Fisher, USA) and 1% (P/S) (stock concentration: 10,000 Units/mL Pen, 10,000 µg/mL Strep, Gibco, Thermo Fisher). Cells at passage 7-9 were used.

*Rheology*

Rheology was performed as previously reported.[1] Briefly, a stress-controlled rheometer (Discovery HR-1, TA Instruments) with an aluminum or steel parallel plate geometry was used (diameter = 40 mm, gap = 500 µm) to measure the mechanical properties of the hydrogels. Polymer solutions were loaded onto the rheometer plate at a rate of 1.0 °C $min^{-1}$ and $T$ = 5 °C, followed by a temperature ramp to $T$ = 37 °C. In the linear viscoelastic (LVE) regime, the moduli were measured at an amplitude of $\gamma$ = 0.02 and a frequency of $\omega$ = 1.0 Hz. Prior to the nonlinear measurements, the polymer solution was allowed to equilibrate for 30 minutes at 37 °C. Here, the hydrogels were subjected to a constant prestress of $\sigma_0$ = 0.5 to 200 Pa, and the differential modulus $K'$ was determined with a small superposed oscillatory stress at frequencies of $\omega$ = 10 to 0.1 Hz (reported data at $\omega$ = 1 Hz). The oscillatory stress was at least 10 times smaller than the applied prestress.

*Encapsulation and conditioned media collection*

hASCs were encapsulated in PIC gels to acquire CM for assessments and functional assays. After sterilization by UV radiation (10 minutes), PIC polymers were dissolved overnight (4°C) in α-MEM medium (10% FCS, 1% P/S) at a concentration of 2 mg/mL. Next, hASCs were harvested (p5) by trypsinization, washed and passed through a 100 µm nylon cell strainer (REF352360, Falcon, USA) to obtain a single-cell suspension (400,000 cells/mL). To prepare cell-gel constructs, cell suspension and polymer solution were mixed thoroughly (1:1) to obtain a final polymer concentration of 1 mg/mL and a final cell density of 200,000 cells/mL. The cell-polymer solution (200 µL) was transferred into a 48-well plate. The plate was then

incubated for 15 minutes (37 °C, 5% $CO_2$) to stabilize the gels. Once gels were formed, 200 µL warm α-MEM medium (10% FCS, 1% P/S) was added on top of cell-gel constructs. As control, a 2D culture was prepared by seeding a tissue culture-treated 48-well plate with 200 (for Multiplex and ELISA) or 400 µL (for wound healing) cell suspension (same total cell number with 3D conditions). The samples were cultured for a total of 7 days. CM were collected at day 1, 3 and 7 and stored at –80 °C. On day 1 and day 3, samples were refreshed with 200 µL fresh medium.

*Wound healing assay*

To assess the effects of CM from stem cells, a wound healing assay was performed with vaginal fibroblasts. Cells were seeded in 2-well culture inserts (80209, Ibidi, Gräfelfing, Germany) that were transferred in a 24-well plate (Corning, tissue culture treated) at a density of 300,000 cells/mL. Once a confluent monolayer was observed (after 24-48 hours of incubation), the 2 Well Culture-inserts were removed, growth medium was aspirated, washed with serum-free medium. Then, CM or α-MEM + 1% P/S with or without a supplement of 10 ng/mL IL-10 (ELISA standard) were added to the wells. To monitor the wound closure, the 24-well plate was placed in the Incucyte live-cell analysis system (Sartorius, Göttingen, Germany) and incubated at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ for 48 hours. Data analysis was performed via the Fiji image analysis software using the MRI wound healing script. The link of the script is http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool.

*Enzyme-linked immunosorbent assay (ELISA)*

The concentrations of IL-10 and VEGF levels in CM were measured by ELISA according to the manufacturer's instructions (Human IL-10/VEGF Mini ABTS ELISA Development kit, Catalog#900-M21/Catalog#900-M10, PeproTech, UK). A high binding 96-well flat bottom plate was used. In short, wells were coated with 100 µL of capture antibody (100 µg/mL) and

incubated at room temperature (RT) overnight. Subsequently, the capture antibody solution was removed and the plate was washed 4× with wash buffer (0.05% Tween-20 in 1× PBS). After the last wash, 300 µL block buffer (1% BSA in 1× PBS) was added to the plate and incubated for at least 1 hour at RT. Followed by aspiration and 4× washing and addition of 100 µL of standard/sample to the plate. Both standard and sample were added in triplicate and incubated for at least 2 hours at RT. Afterwards, all wells were aspirated and washed with PBS (4×), then the detection antibody (100 µL, 100 µg/mL) was added to each well and incubated for 2 hours at RT. Subsequently, the Avidin-HRP conjugate (100 µL) was added after washing with PBS (4×), followed by a 30-minute incubation at RT. Finally, ABTS liquid substrate (100 µL) was added after aspiration and washing with PBS (4×). To monitor the colorimetric process, the optical densities (OD) of the substrate (405 nm) and background (650 nm) were measured using a microplate reader (Spark M10, TECAN, Männedorf, Switzerland).

*Multiplex assays*

Concentrations of chemokines and cytokines in CM were obtained via multiplex (Luminex, Merck, Darmstadt, Germany). A Miliplex Map Human Cytokine/Chemokine Magnetic Bead Panel – Premixed 41 Plex – Immunology Multiplex Assay from Merck (cat#HCYTOMAG-60K) was used. Briefly, sample acquisition was performed according to the user manual provided by the kit used. In summary, for each condition, 25 µL of freshly thawed supernatant medium was used for the measurement of 41 analytes at once using EMD Millipore's Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel. Samples and beads were incubated overnight at 4 °C with shaking at 800 rpm. After washing, a 1-hour incubation with detection antibodies followed by a 30-minute incubation with streptavidin-phycoerythrin was performed. After thorough washing with PBS, concentrations of analytes were read on a Luminex Flexmap 3D$^{(R)}$ with xPONENT$^{(R)}$ Software. Detected median fluorescence intensity (MFI) levels were plotted in BioRad Bio-Plex Manager 6.1 against standards curves diluted in the same matrix as

samples using 5-PL curve fitting. The acceptable recovery range was set to 70%-130%. Values outside this range are expected to be below or above the LLOQ and ULOQ, respectively.

*WST-1 assay*

The metabolic activity of hASCs was determined via the WST-1 cell proliferation assay (Roche, Basel, Switzerland). At day 1, 3 and 7, cell culture media were removed and fresh WST-1 working solution (WST-1 stock: α-MEM =1:9) was added to the wells. Thereafter, the plate was incubated (37 °C, 5% $CO_2$) for 2 hours and all supernatant working solutions were transferred to another 96 well plate. Finally, the absorbance of samples was measured at 450 nm with a plate reader (1420 Multilabel Counter, PerkinElmer, Waltham, USA). Samples were measured in triplicates.

*LIVE/DEAD imaging*

The staining protocol was adapted from the product manual (Invitrogen, Thermo Fisher, USA). In brief, cell culture media on top of gels were gently removed. A 2 µM calcein AM and 4 µM EthD-1 working solution was prepared in pre-warmed PBS and added onto gel samples. After a 30-minute incubation (37 °C, 5% $CO_2$) in the cell incubator, the working solution was replaced by pre-warmed PBS and all samples were imaged by a Zeiss LSM 900 confocal microscope.

*Quant-iT™ PicoGreen™ dsDNA Assay Kit*

DNA concentrations were determined via the PicoGreen DNA quantification assay (P7589, Invitrogen, Carlsbad, USA). After cell culture media were removed, well plates were placed on ice for 20 minutes to liquefy the gels. After 20 minutes, 1 mL cold MilliQ $H_2O$ was added in each well and samples were stored at –80 °C. The quantification was performed according to the product manual. In brief, 60 µL of sample, 70 µL of 1× PicoGreen solution and 100 µL of 1× TE buffer were mixed and added to a white opaque 96-well plate. Subsequently, the plate

was incubated in the dark for 10 minutes at room temperature (RT), after which the fluorescence was measured with a plate reader (PerkinElmer 1420 Multilabel Counter) ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 528 nm). Values were corrected for the difference in sample volumes between 2D and 3D cultures ($volume_{2D}$: $volume_{3D}$ = 1:1.2) during data analysis.

*Statistical analysis*

Data are shown as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) with Tuckey's test was performed for comparison between different groups using Prism Graphpad 7.0. A two-tailed Student's t-test was performed for analysis of variance between two groups. Differences with $P < 0.05$ were regarded as statistically significant.

**Table S1.** Expression of paracrine molecules from 2D and 3D samples determined by Multiplex. The stem cells were encapsulated with a density of 500,000 cells/mL and medium was refreshed every 2 days. The conditioned media from day 6 were measured.

| Factor | Concentration (pg/mL) | | | | |
|---|---|---|---|---|---|
| | Medium control | S1 | S2 | S3 | S4 |
| **EGF** | 27.0 | 24.0 | 33.0 | 37.5 | 32.0 |
| **Eotaxin** | 19.0 | 209.5 | 1028.0 | 30.0 | 569.0 |
| **FGF-2** | 12.0 | 19.0 | 16.0 | 21.0 | 14.0 |
| **FLT-3L** | 15.0 | 16.0 | 35.5 | 26.0 | 28.0 |
| **Fractalkine** | 12.0 | 23.0 | 32.0 | 28.0 | 37.0 |
| **G-CSF** | 23.0 | 29.5 | 32.5 | 31.5 | 35.5 |
| **GM-CSF** | 23.0 | 26.5 | 55.0 | 34.0 | 60.0 |
| **GRO** | 13.5 | 999.0 | 1748.0 | 2820.0 | 3836.5 |
| **INFa-2** | 14.0 | 15.0 | 23.0 | 20.0 | 21.0 |
| **INFy** | 19.0 | 30.0 | 34.0 | 29.5 | 35.5 |
| **IL-10** | 22.0 | 3006.0 | 120.0 | 4903.0 | 268.0 |
| **IL-12p40** | 21.0 | 23.0 | 30.0 | 29.0 | 35.0 |
| **IL-12p70** | 22.0 | 19.0 | 25.5 | 25.0 | 29.5 |
| **IL-13** | 16.0 | 17.0 | 17.0 | 23.0 | 20.0 |
| **IL-15** | 27.0 | 31.5 | 58.0 | 42.0 | 50.0 |
| **IL-17A** | 44.5 | 38.0 | 54.0 | 50.0 | 51.0 |
| **IL-1RA** | 19.0 | 34.0 | 30.0 | 41.0 | 26.5 |
| **IL-1A** | 66.0 | 45.0 | 69.0 | 68.0 | 67.0 |
| **IL-1B** | 25.0 | 66.0 | 85.5 | 114.0 | 89.0 |
| **IL-2** | 22.5 | 32.0 | 30.0 | 34.0 | 36.5 |
| **IL-3** | 25.0 | 23.0 | 26.5 | 30.0 | 35.0 |
| **IL-4** | 19.0 | 17.0 | 21.0 | 25.0 | 27.0 |
| **IL-5** | 20.0 | 21.0 | 28.5 | 30.5 | 33.0 |
| **IL-6** | 36.0 | 33797.5 | 62474.0 | 44617.5 | 59659.0 |
| **IL-7** | 25.0 | 251.0 | 480.5 | 333.0 | 432.0 |
| **IL-6** | 120.0 | 4179.5 | 9830.0 | 12553.0 | 15650.0 |
| **IL-9** | 38.0 | 35.0 | 44.0 | 47.5 | 55.0 |
| **IP-10** | 40.0 | 58.5 | 106.0 | 117.0 | 130.0 |
| **MCP-1** | 15.0 | 37212.0 | 66857.0 | 49404.5 | 59601.5 |
| **MCP-3** | 11.0 | 32.5 | 38.0 | 39.0 | 60.0 |
| **MDC** | 12.0 | 12.0 | 16.0 | 15.0 | 15.0 |
| **MIP-1a** | 22.5 | 22.0 | 28.0 | 26.0 | 28.0 |
| **MIP-1b** | 26.0 | 22.5 | 29.0 | 26.0 | 24.0 |
| **PDGF-AA** | 84.5 | 74.5 | 181.0 | 91.0 | 173.5 |
| **PDGF-AB/BB** | 26.0 | 15.5 | 24.0 | 20.5 | 25.0 |
| **RANTES** | 21.0 | 34.0 | 68.0 | 33.0 | 51.0 |
| **TGF-a** | 49.0 | 37.0 | 44.0 | 55.0 | 56.0 |

| Factor | Concentration (pg/mL) | | | | |
|---|---|---|---|---|---|
| | Medium control | S1 | S2 | S3 | S4 |
| **TNF-a** | 17.0 | 26.0 | 37.5 | 36.0 | 40.0 |
| **TNF-b** | 16.0 | 16.5 | 20.0 | 25.0 | 20.0 |
| **VEGF** | 29.0 | 537.5 | 2836.5 | 1224.0 | 2412.0 |
| **sCD40L** | 15.0 | 14.0 | 16.5 | 17.0 | 19.0 |

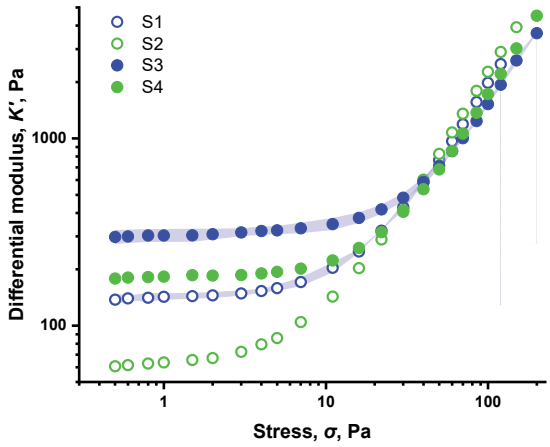


**Figure S1. Differential modulus *K′* as a function of applied stress for S1–S4** (1 mg/mL in α-MEM cell culture medium). The bands in S1 and S3 represent ±standard deviation (n=3), indicating high reproducibility.

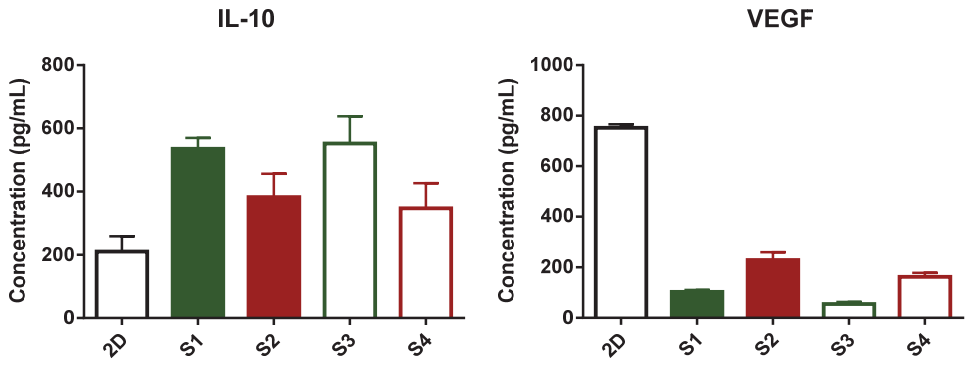


**Figure S2. Absolute concentrations of IL-10 and VEGF determined by ELISA.** The trend in 3D matrices is in line with the results from Multiplex, despite a difference in the absolute values, due to the differences in cell density and detection method.

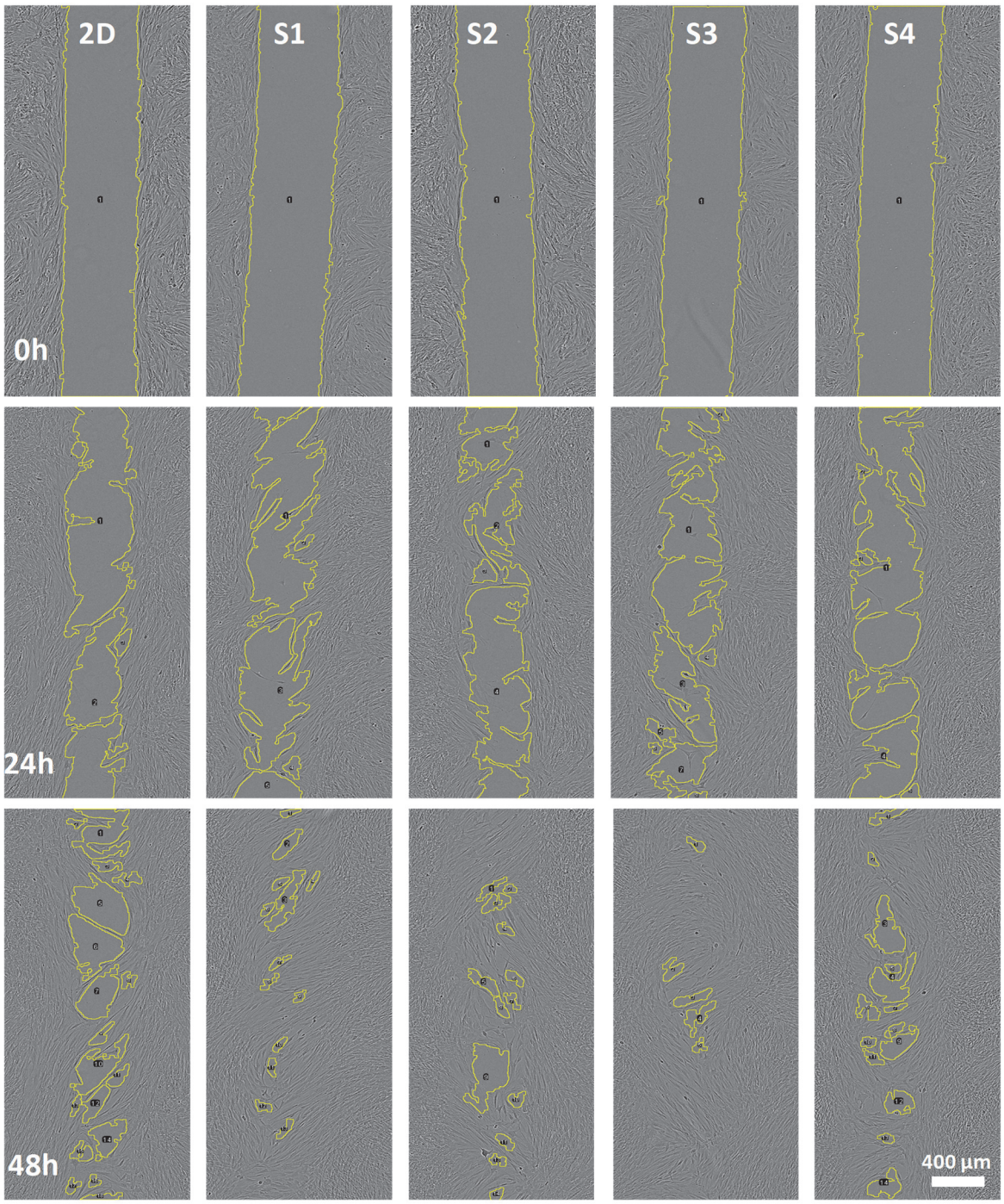


**Figure S3. Bright field images showing the wound closure at different time points.** The outlined areas are acquired by the MRI wound healing script in ImageJ.

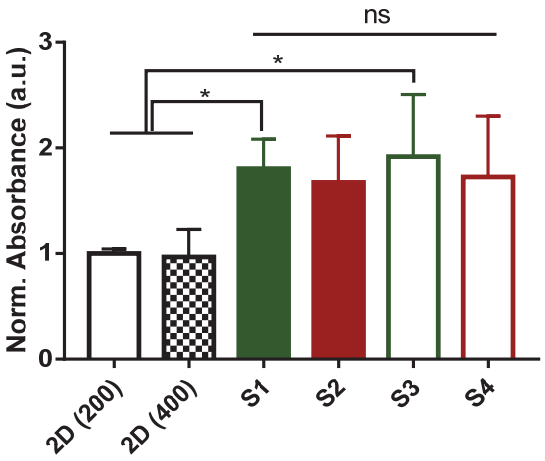


**Figure S4. Proliferation assay on fibroblasts in different conditioned media (CM).** Fibroblasts incubated in all 3D CM show similar proliferation in 48 hours, meanwhile cells in 2D CM show compromised proliferation, regardless of the volume of culture medium used for stem cell culture.

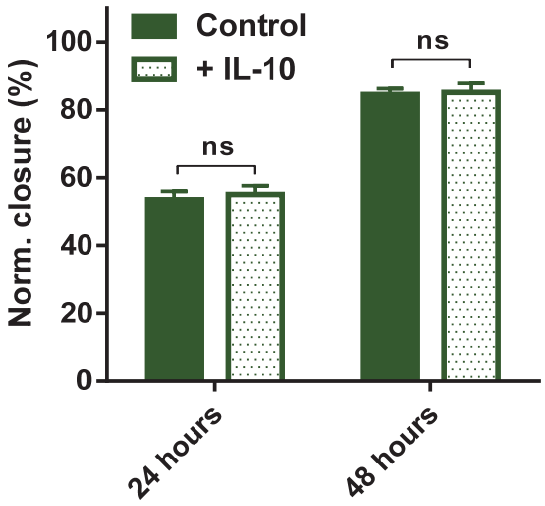


**Figure S5. Addition of IL-10 in low-serum media does not promote wound healing.** The α -MEM medium is supplemented with 1% fetal bovine serum (FCS) instead of a standard 10% FCS.

## References

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