Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting Information

Modeling the tumor microenvironment using chitosan-alginate scaffolds to control the stem-like state of glioblastoma cells

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1. Materials and Methods

1.1. Materials and tissue culture

All cell culture reagents were purchased from Invitrogen unless otherwise noted. All chemicals and polymers were purchased from Sigma and used without further purificaiton unless otherwise noted. The human glioblastoma cell line U-87 MG was purchased from American Type Culture Collection (Manassas, VA) and stably transfected with red fluorescent protein (RFP) as previously reported to generate U-87 MG-RFP. Cells were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen) and 1% antibiotic–antimycotic (Invitrogen) at 37°C and 5% CO_2 in a fully humidified incubator.

1.2. CA scaffold synthesis and surface coatings

Chitosan (practical grade, > 75% deacetylated, MW = 190,000–375,000) and sodium alginate (alginic acid from brown seaweed) powders were used to prepare CA scaffolds as previously reported.[1-5] $CaCl_2$ crosslinked scaffolds were used for coating experiments. CA scaffolds coated with with polycaprolactone (PCL) were prepared as previously reported.[4] Briefly, CA scaffolds were dehydrated in tetrahydrofuran (THF) then added to 10 mg/mL PCL for 2 hr followed by rapid air drying. Coating with HA followed a similar procedure using methanol instead of THF as the solvent. CA scaffolds were dehydrated in methanol then added to 0.2 mg/mL HA in methanol containing 10% DI water with 0.1 M acetic acid. Coated and uncoated scaffolds were sterilized in 70% ethanol for 24 hrs followed by PBS overnight before culturing cells. Uniform coatings on CA scaffolds was confirmed using FTIR and SEM.

1.3. Cell seeding on scaffolds

Cells were seeded into PBS damp CA scaffolds in 12-well plates at 50,000 cells per scaffold in 50 µL of supplemented medium. Cells were allowed to attach to the scaffold for 1 h before adding 1 mL of medium to each well. For 2D cultures, 12-well plates were inoculated with 1 mL medium containing 50,000 cells. Media was replaced every 2 days as required. For co-culture experiments, HUVEC or human astrocyte cells were labeled with Vybrant CFDA SE Cell Tracer dye (Invitrogen) at 10 µM following the manufacturer's protocol. U-87 MG-RFP cells were mixed with CFDA SE cell tracer dye labeled stromal cells at stromal cell:tumor cell ratios of 1:5, 1:1, and 5:1 while keeping the number of tumor cells constant at 50,000 cells per scaffold. Cell mixtures were seeded as described above.

1.4. Live cell fluorescence imaging

Scaffolds in 12-well plates were imaged using an inverted fluorescence microscope (Nikon Instruments, Melville, NY) with the appropriate filters using a Nikon Ri1 Color Cooled Camera System and 10× and 20× objective lenses (Nikon Instruments, Melville, NY).

1.5. Scanning electron microscopy

Scaffolds were fixed with 10% formalin for 24 hr at 4°C. After dehydration by serial washing in increasing ethanol concentrations (0%, 30%, 50%, 70%, 85%, 95%, 100%) with each wash performed twice, samples were critical point dried, mounted, and sputter coated with platinum before imaging with a JSM-7000 SEM (JEOL, Tokyo, Japan).

1.6. PCR

Cells were detached from samples with trypsin, counted, pelleted, and stored at −80ºC before RNA extraction using the Qiagen RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Following reverse transcription (iScript cDNA synthesis kit, Bio-Rad, Hercules, CA), DNA transcripts were probed using BioRad iQ SYBR Green Supermix. A BioRad CFX96 Real-Time Detection System was used for PCR analysis and expression level of CD133 was normalized to β-actin.

1.7. Western blot

Cell pellets were solubilized by incubation for 15 min on ice in 0.1% Triton X-100 in PBS containing 2% β-mercaptoethanol and diluted 1:1 with Laemmli sample loading buffer containing 2% β-mercaptoethanol. After heating at 100ºC for 5 min, 10 µg of extract protein was resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes washed three times with TBS were incubated with 3% QuickBlocker (Chemicon) in TBS for 1 hr at room temperature and then incubated overnight at 4ºC antibody against CD44 (rabbit monoclonal; Abcam, ab51037), Id1 (rabbit monoclonal; Abcam, ab134163), or β-actin (rabbit polyclonal; Abcam, ab75186) in TTBS containing 3% QuickBlocker. Membranes were washed with TTBS before being incubated for 1 hr at room temperature with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Bio-Rad) diluted 1:3000 in 3% QuickBlocker. Membranes were then washed thrice with TTBS and antibody binding visualized by chemiluminescence (Immun-Star detection kit; Bio-Rad) and quantified using the ChemiDoc system running the Quantity One software package (Bio-Rad).

2. Results

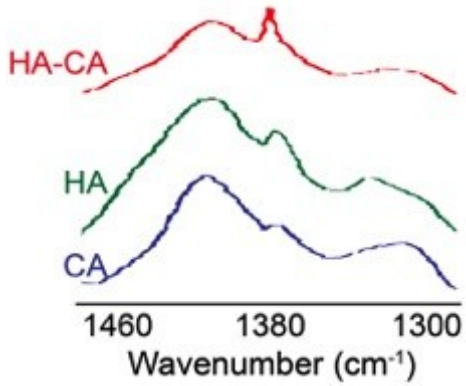

Figure S1. FTIR spectra showing that the representative peak of HA at 1380 cm^{-1} is present on CA-HA scaffolds but is minimal on CA scaffolds.

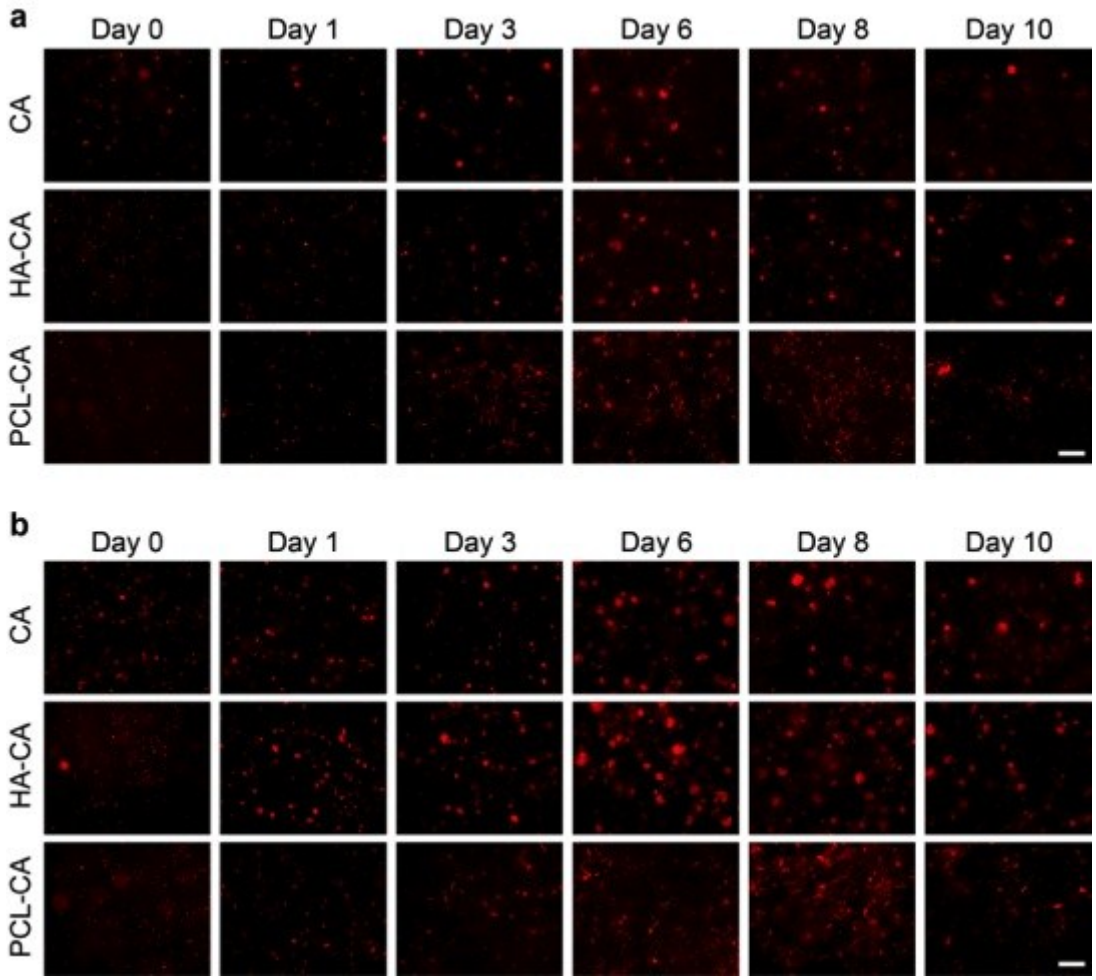

Figure S2. Low magnification images of RFP expressing a) U-118 MG and b) U-87 MG cells in scaffolds over time. Scale bars correspond to 100 µm.

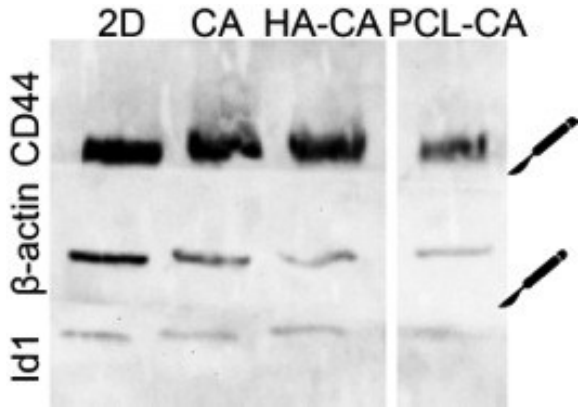

Figure S3. Western blots of U-87 MG cells cultured on 2D, uncoated CA scaffolds, and HA or PCL coated CA scaffolds. Blots were cut and probed independently prior to developing and exposing together.

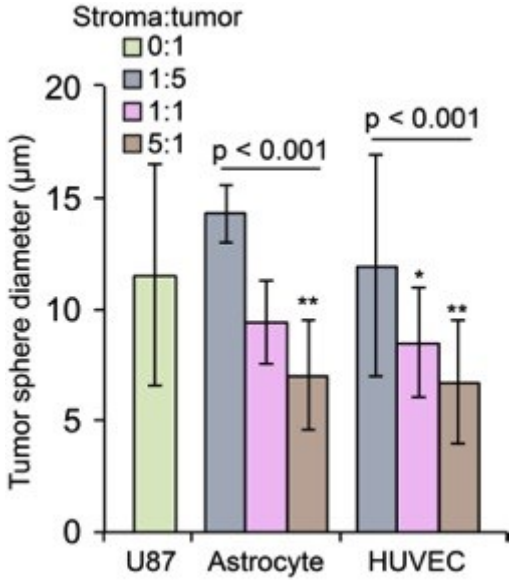

Figure S4. Quantification of tumor sphere diameters from co-culture conditions. Statistical difference in average tumor sphere diameter from U-87 MG only culture (0:1 ratio) was determined by Student's t test (* indicates $p < 0.05$, ** indicates $p < 0.01$). Statistical difference in tumor sphere diameter between different stroma:tumor ratios was determined by one-way ANOVA.

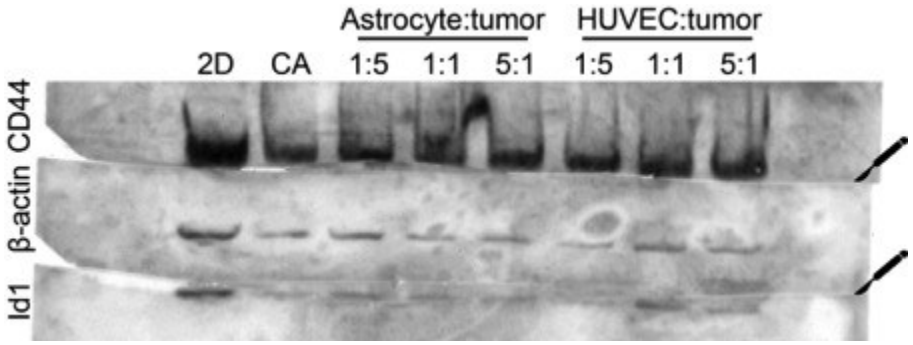

Figure S5. Western blots of U-87 MG cells cultured on 2D, CA scaffolds alone, and co-cultured with human astrocytes or endothelial cells (HUVEC) at different ratios. Blots were cut and probed independently prior to developing and exposing together.

3. References

(1) Florczyk, S. J.; Kim, D. J.; Wood, D. L.; Zhang, M. *J Biomed Mater Res A* 2011, 4, 614-620.

(2) Florczyk, S. J.; Liu, G.; Kievit, F. M.; Lewis, A. M.; Wu, J. D.; Zhang, M. *Advanced healthcare materials* 2012, 5, 590-599.

(3) Kievit, F. M.; Florczyk, S. J.; Leung, M. C.; Veiseh, O.; Park, J. O.; Disis, M. L.; Zhang, M. *Biomaterials* 2010, 22, 5903-5910.
(4) Kievit, F. M.; Florczyk, S. J.; Leung, M. C.; Wang, K.; Wu, J. D.; Silber, J. R.; Ellenbogen, R. G.; Lee, J. S.; Zhang, M. *Biomaterials* 2014, 33, 9137-9143.
(5) Leung, M.; Kievit, F. M.; Florczyk, S. J.; Veiseh, O.; Wu, J.; Park, J. O.; Zhang, M. *Pharm Res* 2010, 9, 1939-1948.