Supporting Information

Biomimetic Material-Assisted Delivery of Human Embryonic Stem Cell Derivatives for Enhanced *In Vivo* Survival and Engraftment

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Content

HA-6ACA synthesis: Carboxylic acid groups of sodium hyaluronate (HA) were reacted with amine groups of 6-aminocaproic acid (6ACA) by N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) coupling reaction (Figure 1A). Briefly, 0.05 g of HA (Mw ~ 48 kDa) was dissolved in 3 mL of MES buffer (pH ~ 4.8, 10 mM) followed by the addition of 0.05 g of EDC (0.264 mmol, 2 mole equivalent of carboxylic acid) and stirred for 20 mins at room temperature. 0.086 g of 6-aminocaproic acid (0.66 mmol, 5 mole equivalent of carboxylic acid) in 4 mL of PBS (pH ~ 7.4, 10 mM) was added to the reaction mixture and stirred for another 12 hrs at room temperature. After completion, the reaction mixture was exhaustively dialyzed for 3 days using a dialysis membrane (MCO ~ 12 kDa) and lyophilized. The dried 6-aminocaproic acid conjugated hyaluronic acid (HA-6ACA) was characterized by ^{1}H NMR and FT-IR, and stored at -20 °C for future use.

Characterization of HA-6ACA by ^{1}H NMR and FT-IR: Fourier transform infrared (FT-IR) spectra were recorded on Nicolet 6700 with Smart-iTR, equipped with liquid nitrogen-cooled MCT-A detector and diamond ATR crystal. The extra peak found at 1691 and 1636 cm^{-1} in HA-6ACA spectrum indicates the amide bond resulting from the coupling reaction between HA and 6ACA, which is not present in the HA spectrum. The peak at 1608 cm^{-1} represents the C=O stretching of sodium salts of carboxylic acids, which is common in both the HA and HA-6ACA spectrum (Figure S1). NMR experiments were carried out on Jeol ECA 500 MHz spectrometer. The peaks at 2.79, 1.29, 1.57 and 1.08-ppm indicate the protons corresponding to the 6ACA molecules grafted to HA.

Docking calculations and clustering analysis**:** The molecular dockings of HA and HA-6ACA on bFGF were performed using the AutoDock Vina 1.1.2 package.[1] We used the crystal structure of bFGF (1BFG) without the bound HDTH and water molecules for docking.[2] A molecular model for two repeat units of HA and HA-6ACA was constructed

using the Vega ZZ 2.3.1.2 package.[3] The 3D coordinates of HA, HA-6ACA, and bFGF were converted into the appropriate format, such as adding polar hydrogens, removing nonpolar hydrogens, and defining rotatable bonds, by using the AutoDock Tools package. In our calculations, we held the bFGF receptor rigid while all rotatable bonds in HA and HA-6ACA were allowed to rotate. Our electrostatic calculations identified a strongly electropositive pocket on the surface of bFGF that was considered as the putative binding location. All docking calculations were limited to a box surrounding this binding location. We were limited to using 2 repeat units of HA and HA-6ACA for the docking calculations due to the steep decrease in the accuracy of Vina's docking algorithm as the number of rotatable bonds in the ligand is increased beyond 30; 2 repeat units on HA-6ACA already possesses 28 rotatable bonds in total. The docking simulations were also carried out with a high exhaustiveness value of 512. Each docking simulation yielded 9 independent configurations with their corresponding binding free energies. We performed 30 such simulations for each of HA and HA-6ACA, yielding a total of 270 configurations for each molecule. We grouped the configurations into clusters containing structurally similar configurations. We used the root-mean-square deviation (RMSD) between the carbon and oxygen atoms of different configurations as a measure of similarity between configurations. The calculated RMSD between all pairs of configurations was used to generate clusters via MATLAB's hierarchical clustering algorithm and each cluster was populated with configurations that did not deviate from each other by an RMSD of more than 3 Å. The docked structures of the bFGF with HA or HA-6ACA complexes were visualized using Pymol and AutoDock Tools.

Electrostatic calculations: We used the APBS package to carry out all electrostatic potential calculations of HA/HA-6ACA and bFGF.[4] The hydrogen atoms were added to the crystal structures using the PDB2PQR program and the charges and radii were assigned according to PARSE force field parameters.[5-6] The electrostatic surface potential of bFGF was obtained by

solving the linearized Poisson-Boltzmann equation (PBE) using the APBS.[4] The calculations were performed at a temperature of 300 K; solute and solvent dielectric constants of 4 and 80; and ion concentration and exclusion radius of 0.2 M and 2.0 Å. The same conditions were also employed when calculating the electrostatic potential of HA and HA-6ACA ligands. APBS output including structures with 3D surface potentials were visualized using both Autodock Tools and PyMol (www.pymol.org).

***Hydrogel synthesis*:** To measure the amount of bFGF adsorbed by HA and HA-6ACA, we have created a crosslinked PEGDA (Mn ~ 508Da) interpenetrated (semi-iPN) with either HA or HA-6ACA molecules as reported elsewhere.[7] Briefly, 0.15 g (w/v) PEGDA was dissolved in PBS solution containing 50 mg ml^{-1} HA and HA-6ACA, respectively. The reaction mixtures were then polymerized using 0.1% (w/v) Irgacure as photoinitiator in BioRad 1mm spacer glass plates. Hydrogels containing 0.15 g (w/v) PEGDA (Mw ~508Da) were synthesized as controls. The hydrogels were cut into discs of 6 mm diameter and used for the ELISA measurements for bFGF and protein adsorption assay.

ELISA measurements: To determine the adsorption of bFGF onto different networks (PEGDA, semi-IPN of PEGDA-HA and PEGDA-HA-6ACA), we have used bFGF ELISA assay kit (RayBiotech, Inc., cat# ELH-bFGF-001) following the manufacturer's protocol. Briefly, equilibrium swollen circular hydrogels measuring 6 mm in diameter were prepared and placed onto a 96-well plate. These hydrogels were incubated with 250 µl of bFGF in PBS (30 ng ml^{-1}) at 37 °C for approximately 1 hr. 100 µl of the supernatant solution was transferred to a bFGF microplate (96-wells coated with anti-human bFGF) and incubated overnight at 4 °C, followed by incubation with a biotinylated antibody and streptavidin solution. After washing, 100 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was added to the wells and samples were incubated for 30 mins. Finally, 50 µl of the stop

solution was added to the samples and their absorbance at 450 nm was measured by using a Multimode Detector (Beckman Coulter, DTX 880). Three biological replicates were used for the measurements. The adsorption was calculated from a standard curve generated by the bFGF standards provided by the manufacturer.

Protein absorption assay: Similarly, to determine the amount of various proteins adsorbed by HA and HA-6ACA, we have used the equilibrium swollen PEGDA, semi-iPN of PEGDA-HA and PEGDA-HA-6ACA hydrogels. The protein adsorptions were determined by using a modified Bradford protein assay (Bio-Rad Protein Assay kit, cat# 500-0006).[8] Briefly, circular hydrogels having a 6 mm diameter were prepared and placed onto 96-well plate. These hydrogels were incubated with 200 µl of collagen type I (BD Biosciences, cat# 354231), collagen type IV (Sigma, cat# C5533), and laminin (Sigma, L6274) solutions of a concentration of 20µg ml^{-1} in PBS for 1 hr at 4 °C. For the collagen type I and IV protein quantification assays, 20 µl of each supernatant solution was mixed with 200 µl of Bradford dye reagent solution, which was prepared by diluting with one part of deionized water and one part of dye solution. For the laminin protein, 20 µl of each supernatant solution was mixed with 200 µl of Bradford dye reagent solution, which was prepared by diluting with four parts of deionized water and one part of dye solution. The solutions were mixed well in a flat-bottom 96-well plate before measuring their absorbance at 595 nm wavelength by using a Multimode Detector (Beckman Coulter, DTX 880). Biological triplicates were used with technical duplicates for the measurements. The adsorption was calculated from a standard curve generated for the corresponding proteins of known concentrations.

Uronic acid assay: All reagents, hyaluronidase (1 TRU $µl^{-1}$), HA, and HA-6ACA solutions (2.5 mg mL^{-1}), were prepared by using a reaction buffer (20 mM sodium acetate, pH ~ 6). To determine the degradation of HA and HA-6ACA, 1.2 ml of HA and HA-6ACA solutions were

mixed with 120 µl of hyaluronidase solution. As controls, the same concentration of HA and HA-6ACA solutions in PBS without hyaluronidase were used. Since the hyaluronidase-mediated degradation of HA into tetrasaccharide and hexasaccharide reaches a steady state in 48 hrs at 37 °C,[9-10] the experimental groups were transferred to a dialysis membrane (MCO ~ 2000 Da) and dialyzed against 2 ml of reaction buffer at 37 °C for 48 hrs. Subsequently, the amount of uronic acid in the reaction buffer, collected from the outside of membrane, was measured through a modified uronic acid assay as described elsewhere.[11] Briefly, 0.2 ml of the collected samples were mixed with 1.2 ml of 12.5 mM tetraborate in concentrated sulfuric acid and heated at 100 °C for 5 mins. After cooling the reaction mixtures in an ice water bath, 20 µl of the m-hydroxydiphenyl reagent (0.15 % m-hydroxydiphenyl in 0.5 % NaOH) was added to each group. The absorbance of the mixture was measured at 520 nm. Since carbohydrates produce a pinkish chromogen in the presence of concentrated sulfuric acid at 100 °C, 0.2 ml of HA solution (2.5 mg mL^{-1}) in the reaction buffer was mixed with 1.2 ml of 12.5 mM tetraborate in concentrated sulfuric acid without adding m-hydroxydiphenyl reagent and used as a blank. The amount of uronic acid, a degradation product of HA, was determined by using solutions of known concentrations of 48 kDA HA as a standard.

***Embryonic Stem Cell Culture*:** HUES9-OCT4-GFP cells were maintained on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells with growth medium containing Knockout DMEM with 10 % KSR (knockout serum replacement), 10 % human plasmanate (Talecris Biotherapeutics), 1 % NEAA (non-essential amino acids), 1 % penicillin/streptomycin, 1 % Gluta-MAX, and 55 µM 2-mercaptoethanol. The medium supplemented with bFGF (30 ng mL^{-1}) was added to cell culture daily.[12] These cells were enzymatically detached using Accutase (Millipore) and routinely passaged when they reach approximately 80 % confluency.

Derivation of mesoderm progenitors expressing PDGFRA: The mesoderm progenitor cells expressing a platelet-derived growth factor receptor-α (PDGFRA) were derived as previously reported.[12] Briefly, undifferentiated HUES9 colonies were dissociated into a single cell suspension by incubating with Accutase for 5 mins at room temperature. Approximately 1.0×10^6 cells were suspended in high glucose DMEM containing 5% FBS, 2 mM L-glutamine, 100 nM dexamethasone, 100 μM hydrocortisone, 1 % penicillin/streptomycin, 10 μM transferrin, 860.9 nM recombinant insulin, 20 nM progesterone, 100.1 μM putrescine, and 30.1 nM selenite (Life Technologies). The cells were cultured in suspension using ultra low attachment plates for 9 days allowing them to form embryoid bodies (EBs). The medium was changed every alternative day. The EBs were transferred to a 10 cm cell culture dish, which was precoated with growth factor-reduced Matrigel (1:25 diluted in KnockOut DMEM; BD Biosciences), using a 1:6 split ratio. The attached EBs were cultured with the afore-mentioned medium for an additional 7 days until a larger number of cells migrated out of the EBs. These migrating cells were enzymatically detached by trypsin and filtered through a 40 μm cell strainer. The isolated cells were sorted for a $PDGFRA^+/OCT4\text{-}GFP^-$ ($PDGFRA^+$ cell) population by FACS. To FACS isolate PDGFRA expressing cells, the hESC-derived cells were enzymatically detached and resuspended in a buffer solution [2 % FBS and 0.09 % sodium azide in DPBS (BD Biosciences)]. The cells were stained with either Alexa Fluor 647-conjugated PDGFRA or Alexa Fluor 647-conjugated mouse IgM,κ isotype control antibodies (Biolegend) on ice for 30 mins. After the staining, cells were washed, resuspended in the buffer solution, followed by cell sorting using BD Biosystems FACSCanto. Data were analyzed with the CellQuest Pro software. The $PDGFRA^+$ cells were expanded in high glucose DMEM supplemented with 10 % FBS, 2 mM L-glutamine, and 1 % penicillin/streptomycin and expanded *in vitro*. Passage 8 cells were used for the animal studies.

***Preconditioning of myogenic progenitor cells before transplantation*:** The hESC-derived PDGFRA^{+} cells were plated onto tissue culture plates with a seeding density of 1×10^4 cells $(cm^2)^{-1}$ and cultured in an induction medium containing high glucose DMEM supplemented with 2 mM L-glutamine, 100 nM dexamethasone, 100 μM hydrocortisone, 1 % penicillin/streptomycin, 10 μM transferrin, 860.9 nM recombinant insulin, 20 nM progesterone, 100.1 μM putrescine, and 30.1 nM selenite with 10 % FBS for 14 days. The induction medium was changed every other day.

***Cell transplantation*:** Animal experiments were performed according to the protocols approved by Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego. Twenty four hrs prior to cell transplantation, TA muscles of 2-month-old immune-deficient NOD.CB17-Prkdc^{scid}/J mice were injured by intramuscular injection of 30 μL carditoxin (0.5mg mL^{-1}; Sigma, cat# C9759). Right before cell transplantation, NOD/SCID mice were administered with ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) and approximately 3.0 x 10^5 PDGFRA^{+} cells cultured for 14 days in induction medium were resuspended in 10 μL of either physiological saline solution, HA (30 mg mL^{-1}) or HA-6ACA solution (10, 30, and 50 mg mL^{-1}), and directly injected to the TA muscles. Upon 14 days post-transplantation, all TA muscles were harvested and embedded in Optimal Cutting Temperature compound (OCT) for cryosectioning. Survival and engraftment of the transplanted cells were histologically evaluated.

***Immunofluorescence staining*:** Immunofluorescence staining was performed using the following primary antibodies: PAX7 (1:5; Developmental Studies Hybridoma Bank), human lamin A/C (1:100; Vector Laboratories), and mouse laminin (1:200; Millipore). The following secondary antibodies were used: goat anti-mouse Alexa 488 (1:250; Life Technologies), and

goat anti-rabbit Alexa 546 (1:200; Life Technologies). The TA samples were embedded in OCT for cryosectioning. The frozen tissue blocks of the TA muscles were sectioned into 20 µm thick sections using a cryostat (Leika CM 3050) in the longitudinal plane. For immunofluorescence staining, samples were briefly washed in PBS to remove OCT, followed by fixing in 2 % PFA for 8 mins at room temperature. Immediately before staining, the sections were blocked using a blocking buffer containing 0.3 % Triton X-100 and 20 % normal goat serum in PBS for 1 hr at room temperature. Samples were stained with human lamin A/C and mouse laminin for overnight at 4 °C, followed by 3 sequential 10 mins washes in PBS. Sections were then incubated with secondary antibodies for 1 hr at room temperature. The nuclei were stained with Hoechst 33342 (2 mg ml^{-1}; Life Technologies) for 5 mins at room temperature. For PAX7 staining, antigen retrieval was performed.[13] Briefly, the sections were first stained with primary human lamin A/C antibody and corresponding secondary antibody. Next, samples were post fixed with 2% PFA for 8 mins at room temperature and immersed in preheated (90 °C) 100 mM citric acid (pH ~ 6) for 15 mins, followed by 3 sequential washes with PBS for 5 mins each time. The sections were then incubated with PAX7 antibody, followed by incubating with secondary antibody for 1 hr at room temperature. Imaging was performed using a fluorescence microscope (Carl Zeiss; Axio Observer A1).

***Image analysis*:** Total number of transplanted cells (lamin A/C^+ cells) in the host tissue was quantified using NIH ImageJ software. The images were filtered and adjusted for threshold for quantifying the total number of donor cells found in the host tissues. The number of donor cells fused with the host myofibers were counted manually. Three serial sections were analyzed per muscle sample for each of the biological triplicates. The percentage of transplanted cells integrated with the host myofibers was presented as a ratio of the total number of lamin A/C^+ cells fused with the myofibers to the total number of lamin A/C^+ cells. Similarly, for each of the three biological replicates, within each muscle sample three serial

sections were analyzed and the number of donor cells contributing to the satellite cell compartment ($PAX7^+$ cells) were determined manually. The percentage of PAX7 positive cells was represented as a ratio of total number of PAX7 positive cells found underneath the basal lamina to the total number of lamin A/C^+ cells.

***Statistical analysis*:** All values were presented as mean ± standard deviation and statistical significance was determined by single-factor analysis of variance (ANOVA) with Tukey's Multiple Comparison Test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0001$).

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Supporting Figures and Figure Legends

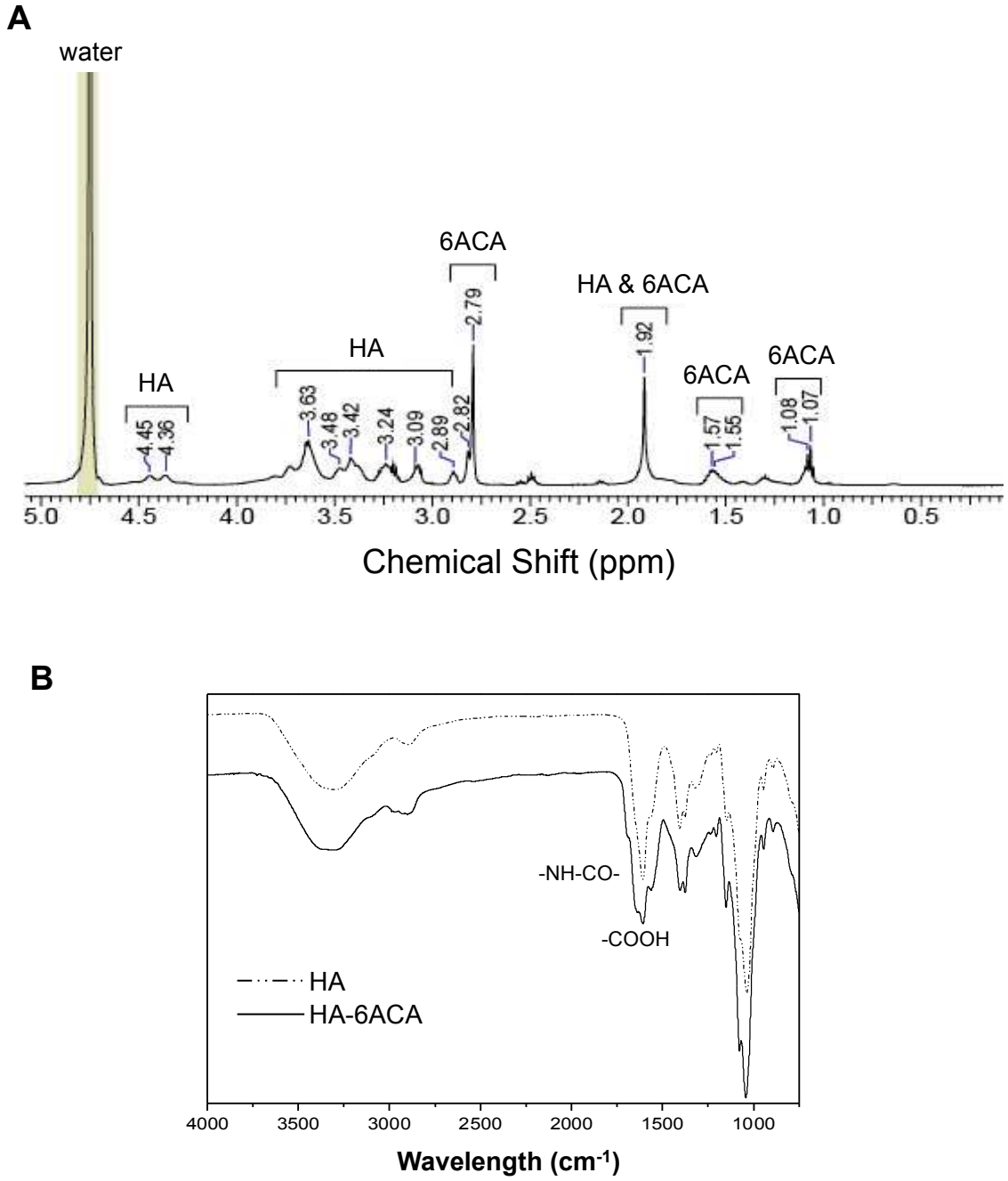

Figure S1. (A) Characterization of HA-6ACA by ^{1}H NMR. **(B)** FTIR spectra of HA and HA-6ACA.

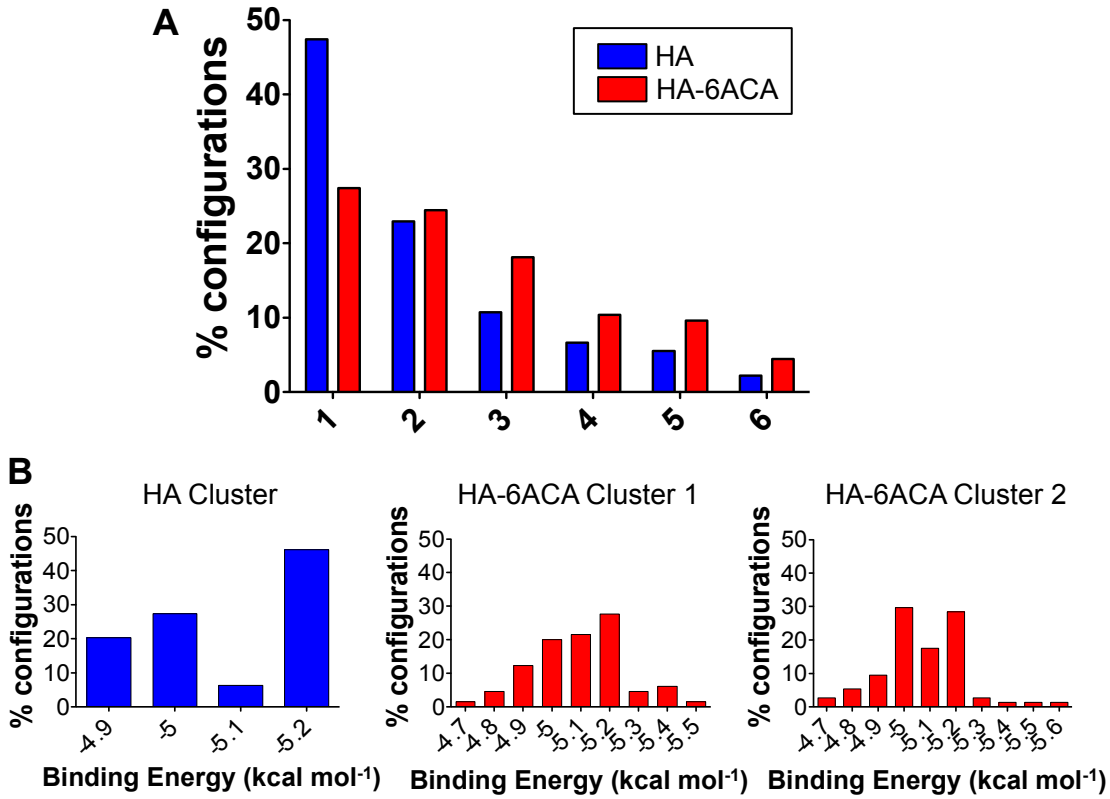

Figure S2. (A) Percentage population of 6 most populated clusters of HA and HA-6ACA binding to bFGF. Majority of HA binding configurations fall into the most populated cluster, while HA-6ACA binding configurations were distributed amongst smaller clusters. **(B)** Distribution of the binding free energy of the most populated cluster(s) of HA and HA-6ACA. Only the two largest clusters for HA-6ACA that are equally populated are presented. The fraction of configurations exhibiting the lowest energy for HA-6ACA (~1-5%) is much smaller than that for HA (~45%) due to the additional 6ACA side chains in the former. Significantly longer docking calculations are required to sample the additional rotational degrees of freedom resulting from these side chains. All energy distributions exhibit a peak at –5.2 kcal mol^{-1}, likely due to both types of molecules exhibiting similar backbone configurations in their most favorable docked configurations. The 6ACA side chains likely bind to bFGF in tight pockets and these configurations are not easy to access computationally. Thus, few docking solutions lead to these favorable side chain configurations exhibiting binding energies of –5.5 kcal mol^{-1} and –5.6 kcal mol^{-1}.

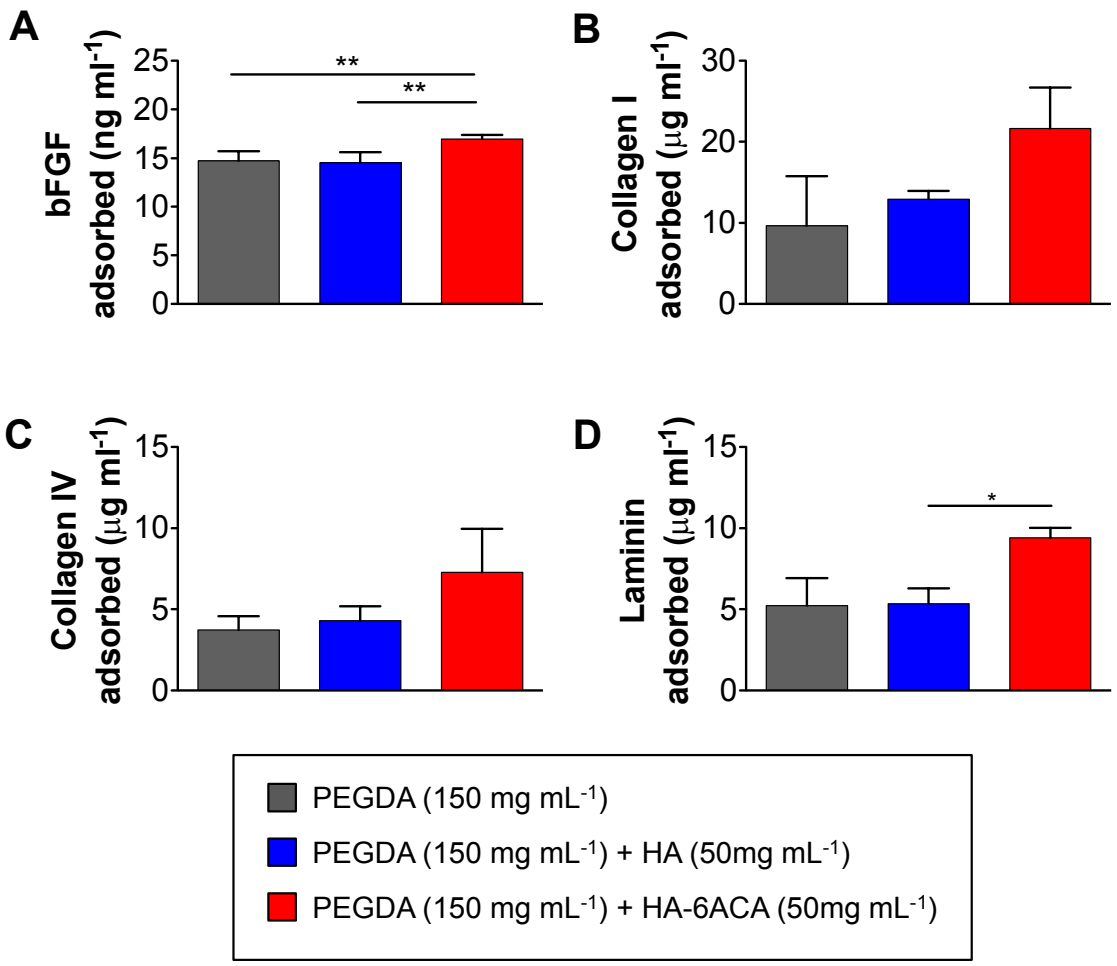

Figure S3. (A) Adsorption of bFGF onto different semi-IPN networks. Quantification of ECM proteins, **(B)** collagen type I, **(C)** collagen type IV, and **(D)** laminin.

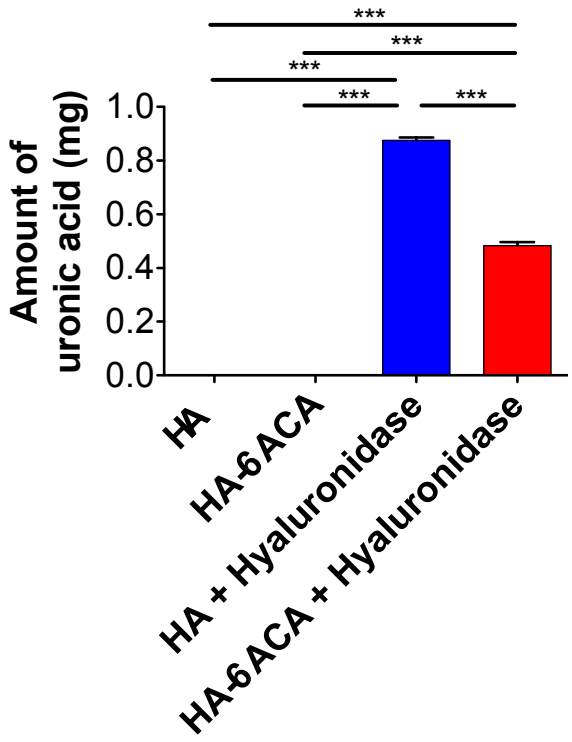

Figure S4. Degradation profile of HA and HA-6ACA in PBS with and without hyaluronidase.

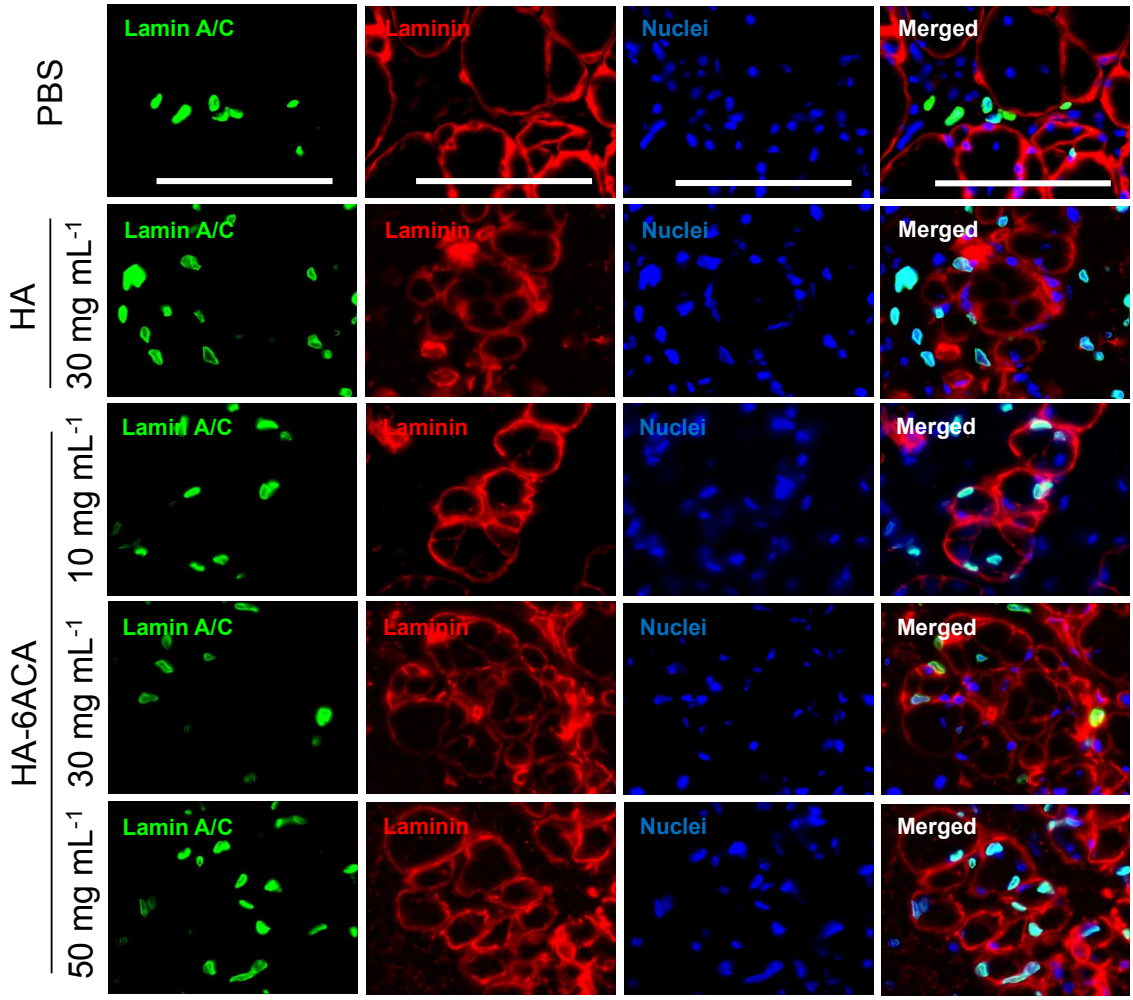

Figure S5. Skeletal muscle tissues transplanted with hESC-derived progenitor cells. Immunofluorescence staining for human-specific lamin A/C (green), laminin (red), and nuclei (blue). Scale bar = 50 μm.