

Supplementary Information

Enhanced Articular Cartilage by Human Mesenchymal Stem Cells in Enzymatically Mediated Transiently RGDS-Functionalized Collagen-Mimetic Hydrogels

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Synthesis and purification of MMP7-cleavable, non-cleavable ScrMMP7, and cyclic RGDS peptides.

The MMP7 (PLELRA)-cleavable and non-cleavable scrambled MMP7 (ScrMMP7; PALLRE) peptides were synthesized manually on a 2 mmol scale using standard Fmoc solid phase peptide synthesis techniques as previously described [34]. For each coupling, the Fmoc protecting group was removed with 20% (v/v) piperidine in DMF followed by washing with DCM and DMF. Amino acids were activated by adding 4 molar equivalent of each Fmoc protected amino acid to 3.95 molar equivalent of HBTU and dissolved in DMF. Six molar equivalent of DIEA was added to the amino acid solution and the coupling solution added to the resin. The coupling reaction was allowed to proceed for 120–180 min before the resin was washed in DCM and DMF. Ninhydrin tests were performed after each Fmoc deprotection and coupling step to monitor the presence of free amines.

Two Fmoc-Lys(Mtt)-OH were also coupled to the peptides at the N- and C- termini each using methods described above. The ninhydrin test for free amines was conducted at room temperature for 30 min since the Mtt protecting group is heat-labile. The Mtt protecting group was removed from the amine on the lysine side chain with 5% (v/v) TFA in DCM for 2 min on the shaker. The solution turns bright yellow as the Mtt is removed, and this was repeated at least 5 times or until the solution was clear. The beads were washed thoroughly with DCM and DMF before running the ninhydrin test at room temperature for 30 min. To attach acrylate groups to the peptide, acryloyl chloride was activated by adding 10 molar

equivalents of acryloyl chloride and 10 molar equivalents of DIEA in DCM. The coupling was verified with the ninhydrin test at room temperature for 30 min.

Once the synthesis was completed, the peptides were cleaved in 95% (v/v) trifluoroacetic acid (TFA), 2.5% (v/v) triisopropyl silane (TIS), and 2.5% (v/v) H₂O for 240 min. TFA was removed using rotary evaporation, and the peptide residues were precipitated and washed with cold diethyl ether by centrifugation. The peptide precipitates were then allowed to dry under vacuum to remove residual ether. The peptides were purified (Fig. S1) using reversed phase preparative high performance liquid chromatography (HPLC; Shimadzu) in an acetonitrile/water gradient under acidic conditions on a Phenomenex C18 Gemini NX column (5 µm pore size, 110 Å particle size, 150 x 21.2 mm). Following purification, the peptides were lyophilized on a freeze dryer (Labconco) for storage prior to use. The purified peptide masses were verified by matrix assisted laser desorption spectroscopy (MALDI; Waters).

A cyclic RGDS peptide (GRGDSC) was synthesized at a 1 mmol scale on a 2-chlorotriyl chloride resin (100–200 mesh; VWR). Fmoc-Cys(Trt)-OH (Novabiochem) was dissolved at 1 molar equivalent in DCM with a small amount of DMF added until fully solubilized. Half the solution was added to the resin along with 500 µL of DIEA for 15 min shaking on a wrist action shaker. This was repeated with the remaining solution followed by thorough washing with DMF and DCM. A ninhydrin test was performed to monitor the coupling by detecting the presence of free amines. The remaining free amines were capped by adding a solution of 5% (v/v) acetic anhydride with 2.5% (v/v) DIEA in DMF for 10 min with shaking, and repeated with 5 min shaking using fresh solution. The resin was then washed thoroughly with DCM and DMF before the ninhydrin test. The Fmoc protecting group was removed as described above and Fmoc-Asp(OtBu)-Ser(psiMe,Mepro)-OH (Merck) was coupled at 2 molar equivalents with 1.95 molar equivalents of HBTU and 3 molar equivalents of DIEA in DMF. The remaining free amines were capped, and all other amino acids were coupled as described above. The protected peptide was cleaved from the resin by adding 10 mL of 5% (v/v) TFA in DCM for 10 min with shaking. The solution was drained into a round

bottom flask and the resin rinsed with DCM until the solution in the synthesis vessel was clear. The DCM and TFA were removed carefully by rotary evaporation, leaving approximately 40 mL of solution to avoid cleaving the protecting groups from the peptide. Ammonium hydroxide (10 mL) was added to neutralize the TFA followed by acetonitrile to increase peptide solubility. The protected peptide was purified by reverse phase preparative HPLC running a mobile phase gradient of 80% ultrapure H₂O and 20% (v/v) ACN to 100% (v/v) ACN with 0.1% (v/v) TFA. The solvent was removed by rotary evaporation until the protected peptide was completely dry, and then re-dissolved in DMF at 1 mg/mL. The peptide was cyclized by adding 2 equivalents of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop; AGTC Bioproducts) and 3 equivalents of DIEA overnight. The DMF was removed by rotary evaporation and the remaining product was re-dissolved in acetonitrile/water until solubilized and purified by reverse phase preparative HPLC as described above. The remaining protecting groups were removed using 95% (v/v) TFA with 2.5% (v/v) H₂O and 2.5% (v/v) TIS. The peptide was precipitated in cold DEE and purified by reverse phase preparative HPLC. Liquid chromatography-mass spectrometry (LC-MS) was performed on an Agilent 6130 Quadrupole LC-MS coupled to an Agilent 1260 Infinity LC using a 150 x 4.6 mm Phenomenex Gemini NX C18 column with a 5 μm pore size and 100 Å particle size. Ultrapure H₂O and acetonitrile each containing 0.1% (v/v) formic acid (VWR) by volume were used for the mobile phase at a flow rate of 1 mL/min. The peptide was eluted with a gradient of 95% (v/v) H₂O to 95% (v/v) acetonitrile over 11 min. The electrospray source was operated with a capillary voltage of 3.2 kV and a cone voltage of 25 V with nitrogen used as the nebulizer and de-solvation gas at a total flow of 600 L/h. Although cyclic GRGDSC did not stick to the column, electrospray ionization (ESI) of an early elution confirmed the correct mass.

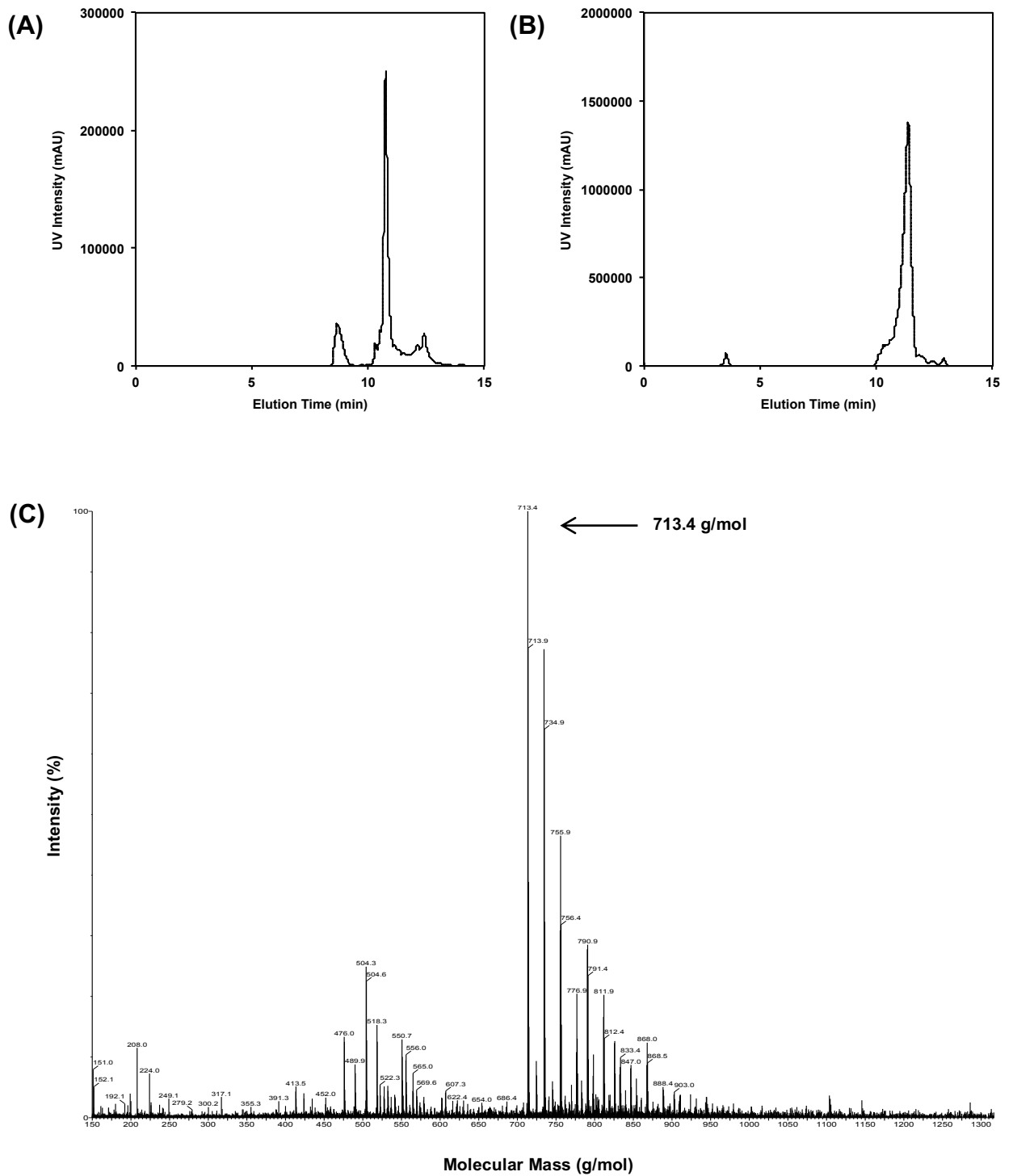
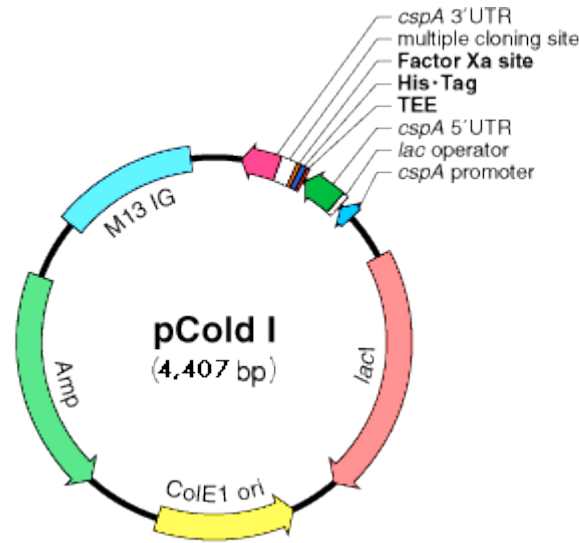


Fig. S1. Representative reversed phase high performance liquid chromatography (HPLC) chromatograms and electrospray ionization (ESI) spectra of (A, C) MMP7 (MW/2: 713.4 g/mol, MW: 1426.8 g/mol) and (B) ScrMMP7 (MW/2: 713.4 g/mol, MW: 1426.8 g/mol) peptides, respectively.



pCold I vector sequence:

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1 AAGGAATGGT GTGGCCGATT AATCATAAAT ATGAAAAATA ATTGTTGCAT CACCCGCCAA TCGTGGCTT AATGCACATC AAATTGTGAG CGGATAACAA

      BmtI
      -----+
      NheI
      +-----
101 TTTGATGTGC TAGCGCATAT CCAGTGTAGT AAGGCAAGTC CCITCAAGAG TTATCGTTGA TACCCCTCGT AGTGCACATT CCTTTAACGC TTCAAAATCT
201 GTAAAGCAGC CCATATCGCC GAAAGGCACA CTTAATTATT AAGAGGTAAT ACACCATGAA TCACAAAGTG CATCATCATC ATCATCATAT CGAAGGTAGG

      AbsI
      -----+
      KpnI
      -----+
      PspXI
      -----+
      XhoI
      -----+
      EcoRI
      -----+
      SalI
      -----+
      NdeI
      -----+
      Acc65I
      -----+
      BamHI
      -----+
      HindIII
      -----+
      BspMI
      -----+
      XbaI
      -----+
301 CATATGGAGC TCGGTACCCT CGAGGGATCC GAATTC AAGC TTGTCGACCT GCAGTCTAGA TAGGTAATCT CTGCTTAAAA GCACAGAATC TAAGATCCCT

      ClaI
      -----+
401 GCCATTTGGC GGGGATTTTT TTATTTGTTT TCAGGAAATA AATAATCGAT CGCGTAATAA AATCTATTAT TATTTTTGTG AAGAATAAAT TTGGGTGCAA

      BsmI
      -----+
      EcoO109I
      -----+
      NspI
      -----+
501 TGAGAATGCG CAGGCCCTTT CGTCTCGCGC GTTTCGGTGA TGACGGTGAA AACCTCTGAC ACATGCAGCT CCCGGAGACG GTCACAGCTT GTCGTGAAGC
601 GGATGCCGGG AGCAGACAAG CCCGTCAGGG CGCGTCAGCG GGTGTTGGCG GGTGTCGGGG CTGGCTTAAC TATGCGGCAT CAGAGCAGAT TGTACTIONGAG
701 GTGCACCATA AAATTGTAAG CGTTAATATT TTGTTAAAAA TCGCGTTAAA TTTTGTGTTA ATCAGTTCAT TTTTAAACCA ATAGCCGAAA ATCGGCAAAA

      PstI
      -----+
801 TCCCTTATAA ATCAAAAGAA TAGCCCGAGA TAGGGTTGAG TGTTGTTCCA GTTTGGAACA AGAGTCCACT ATTAAGAAGC GTGGACTCCA ACGTCAAAGG

      BsaAI
      -----+
901 GCGAAAAACC GTCTATCAGG GCGATGGCCC ACTACGTGAA CCATCACCCA AATCAAGTTT TTTGGGGTCG AGGTGCCGTA AAGCACTAAA TCGGAACCC

      NaeI
      -----+
      NgoMIV
      -----+
1001 AAAGGGAGCC CCGGATTTAG AGCTTGACGG GGAAGGCCGG CGAACGTGGC GAGAAAGGAA GGGAAAGAAAG CGAAAGGAGC GGGCGCTAGG GCGCTGGCAA
1101 GTGTAGCGGT CACGTCGCGC GTAACCACCA CACCCGCCCG GCITTAATGCG CCGCTACAGG GCGCGTACTA TGTTTGCTTT GACGTATGCG GTGTGAAATA
1201 CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGTC AGGTGGCACT TTTGGGGGAA ATGTGCGCGG AACCCCTATT TGTTTTTTTT TCTAAATACA
1301 TTCAAATATG TATCCGCTCA TGAGACAATA ACCCTGATAA ATGCTTCAAT AATATTGAAA AAGGAAGAGT ATGAGTATTC AACATTTCCG TGTCGCCCTT
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      -----+
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      ScaI
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1801 AGGAGCTAAC CGTTTTTTTG CACAACATGG GGGATCATGT AACTGCGCTT GATCGTTGGG AACCGGAGCT GAATGAAGCC ATACCAACAG ACGAGCGTGA
1901 CACCACGATG CCTGTAGCAA TGGCAACAAC GTTGCAGAAA CTATTAACGT GCGAAGTACT TACTCTAGCT TCCCGGCAAC AATTAATAGA CTGGATGGAG

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2101 TTGCAGCACT GGGGCCAGAT GGTAAAGCCCT CCGGTATCGT AGTTATCTAC ACGACGGGGA GTCAGGCAAC TATGGATGAA CGAAATAGAC AGATCGCTGA
2201 GATAGGTGCC TCACTGATTA AGCATTGGTA ACTGTGAGAC CAAGTTTACT CATATATACT TTAGATTGAT TAAAAAATTC ATTTTAAATT TAAAAGGATC
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2401 CTTGAGATCC TTTTTTTCG CGCGTAATCT GCTGCTTGCA AACAAAAAAA CCACCGCTAC CAGCGGTGGT TTGTTTGCCG GATCAAGAGC TACCAACTCT
2501 TTTTCCGAAG GTAACCTGGT TCAGCAGAGC GCAGATACCA AATACTGTTC TTCTAGTGTA GCCGTAGTGA GGCCACCACT TCAAGAATCT TGTAGCACC
                AlwNI
                -----+
2601 CCTACATACC TCGCTCTGCT AATCCTGTTA CCAGTGGCTG CTGCCAGTGG CGATAAGTCG TGCTTTACCG GGTGGGACTC AAGACGATAG TTACCGGATA
2701 AGGCGCAGCG GTCGGGCTGA ACGGGGGGTT CGTGACACACA GCCCAGCTTG GAGCGAACGA CCTACACCGA ACTGAGATAC CTACAGCGTG AGCTATGAGA
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2901 TGGTATCTTT ATAGTCTCTG CGGGTTTCGC CACCTCTGAC TTGAGCGTGC ATTTTGTGTA TGCTCTGACG GGGGGCGGAG CCTATGGAAG AACGCCAGCA
3001 ACGCGGCCCT TTTACGGTTC CTGGCCTTTT GCTGGCCTTT TGCTCACATA GTCATGCCCC GCGCCACCGG GAAGGAGCTG ACTGGGTTGA AGGCTCTCAA
3101 GGGCATCGGT CGAGATCCCG GTGCCTAATG AGTGAAGTAA CTTACATTA TGGCGTTGCG CTCACTGCCG GCTTTCAGT CGGGAAACCT GTCGTGCCAG
                BbeI
                -----+
                SfoI
                -----+
                NarI
                -----
                KasI
                -----
3201 CTGCATTAAT GAATCGGCCA ACGCGCGGGG AGAGGCGGTT TGCCTATTGG GCGCCAGGST GGTTTTTCTT TTCACCAGTG AGACGGGCAA CAGCTGATTG
                HpaI
                -----+
3301 CCCTTCACCG CCTGGCCCTG AGAGAGTTGC AGCAAGCGGT CCACGCTGGT TTGCCCCAGC AGGCGAAAAA CCTGTTTGAT GGTGGTTAAC GGCGGGATAT
                EcoRV                      BssHII
                -----+                      -+-----
3401 AACATGAGCT GTCTTCGGTA TCGTCGTATC CCACTACCGA GATATCCGCA CCAACGCGCA GCCCGGACTC GGTAATGGCG CGCATTGGCG CCAGCGCCAT
3501 CTGATCGTTG GCAACCAGCA TCGCAGTGGG AACGATGCCG TCATTCAGCA TTTGCATGGT TTGTGAAAAA CCGGACATGG CACTCCAGTC GCCTTCCCGT
                ApaI
                -----+
                PspOMI
                -----
3601 TCCGCTATCG GCTGAATTTG ATTGCGAGTG AGATATTTAT GCCAGCCAGC CAGACGCAGA CGCGCCGAGA CAGAACTTAA TGGGCCCGCT AACAGCGCGA
                BstEII
                -+-----
3701 TTTGCTGGTG ACCCAATGCG ACCAGATGCT CCACGCCAG TCGCGTACCG TCTTCATGGG AGAAAAAAT ACTGTTGATG GGTGTCTGGT CAGAGACATC
                BclI                      AflIII
                -+-----                      -+-----
                MluI
                -+-----
3801 AAGAAATAAC GCCGGAACAT TAGTGCAGGC AGCTTCCACA GCAATGGCAT CCTGGTCATC CAGCGGATAG TTAATGATCA GCCCACTGAC GCGTTGCGCG
3901 AGAAGATTGT GCACCCCGCG TTTACAGGCT TCGACGCCCG TTCGTTCTAC CATCGACACC ACCACGCTGG CACCCAGTTG ATCGGCGCGA GATTTAATCG
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4101 AATGTAATTC AGCTCCGCCA TCGCCGCTTC CACTTTTTTC CGCGTTTTTC CAGAAACGTG GCTGGCCTGG TTCACCACCG GGGAAACGGT CTGATAAGAG
                BstAPI
                -----+
4201 ACACCGGCAT ACTCTGCGAC ATCGTATAAC GTTACTGGTT TCACATTAC CACCCTGAAT TGACTCTCTT CCGGGCGCTA TCAIGCCATA CCGCGAAAGG
                EcoNI
                -----+
4301 TTTTGGCGCA TTCGATGGTG TCCGGGATCT CGACGCTCTC CCTTATGCGA CTCCTGCATT AGGAAGCAGC CCAGTAGTAG GTTGAGGCCG TTGAGCACCG
4401 CCGCCGC

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Fig. S2. A fully annotated plasmid map of the pColdI vector system used to sub-clone the final DNA sequence for expression in *E. coli*.

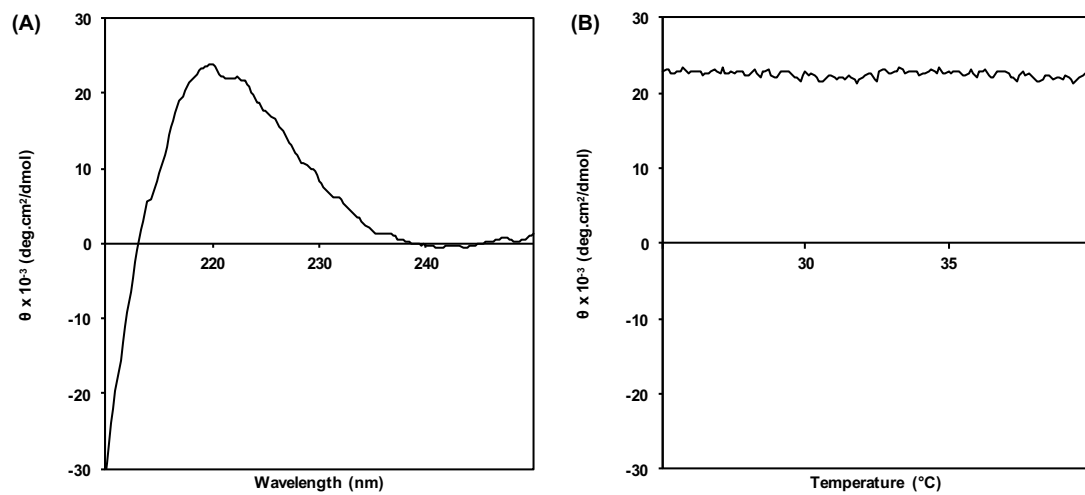


Fig. S3. Secondary structure of Scl2 proteins. (A) The ellipticity of the functionalized Scl2 proteins monitored from 210 to 250 nm at 37 °C. (B) The ellipticity of the functionalized Scl2 proteins monitored at 220 nm from 25 to 40 °C.

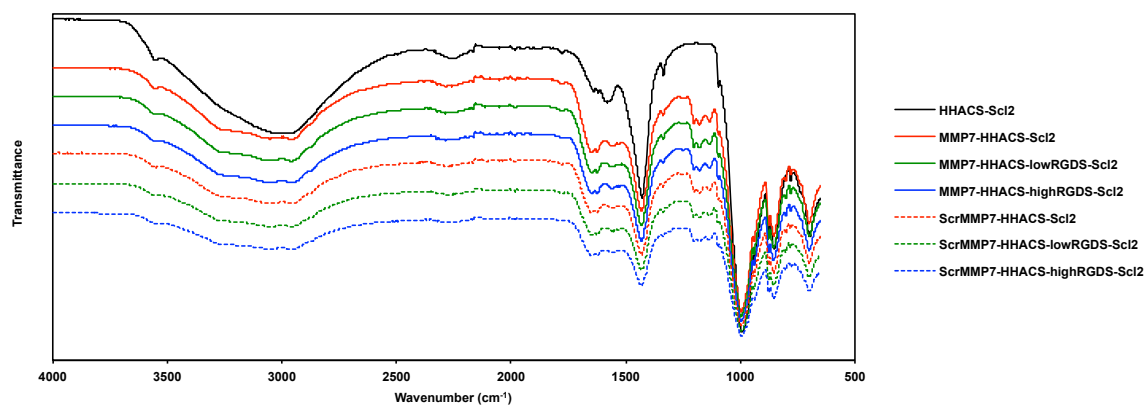


Fig. S4. Representative Fourier transform infrared (FTIR) spectra of functionalized Scl2 proteins confirming the conjugation of Scl2 with acrylate-functionalized MMP7-cleavable and non-cleavable ScrMMP7 peptides.

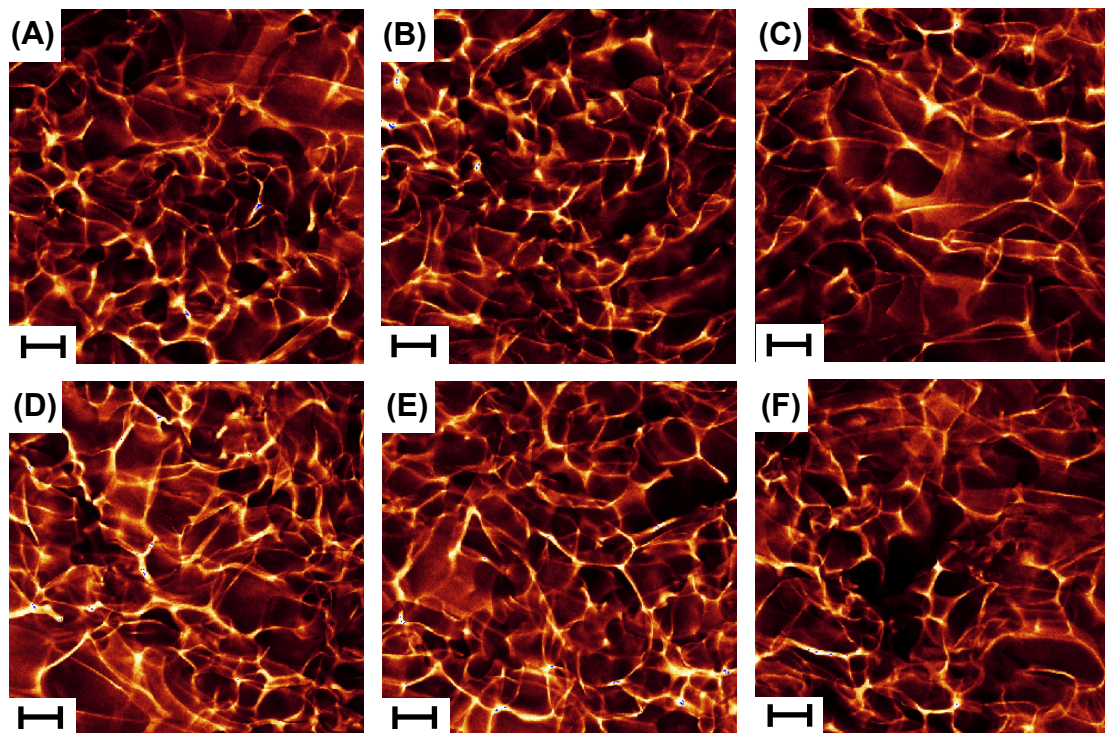


Fig. S5. Representative multi-photon second harmonic generation (MP-SHG) images of acellular (A) MMP7-HHACS-Scl2, (B) MMP7-HHACS-lowRGDS-Scl2, (C) MMP7-HHACS-highRGDS-Scl2, (D) ScrMMP7-HHACS-Scl2, (E) ScrMMP7-HHACS-lowRGDS-Scl2, and (F) ScrMMP7-HHACS-highRGDS-Scl2 hydrogels (scale bars are 10 μm).

- hMSCs + hMSCs

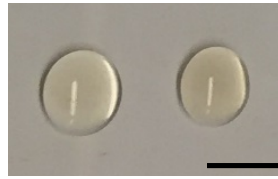


Fig. S6. Representative photos of acellular and hMSC-seeded MMP7-HHACS-Sc12 hydrogel constructs (scale bars are 1 cm).

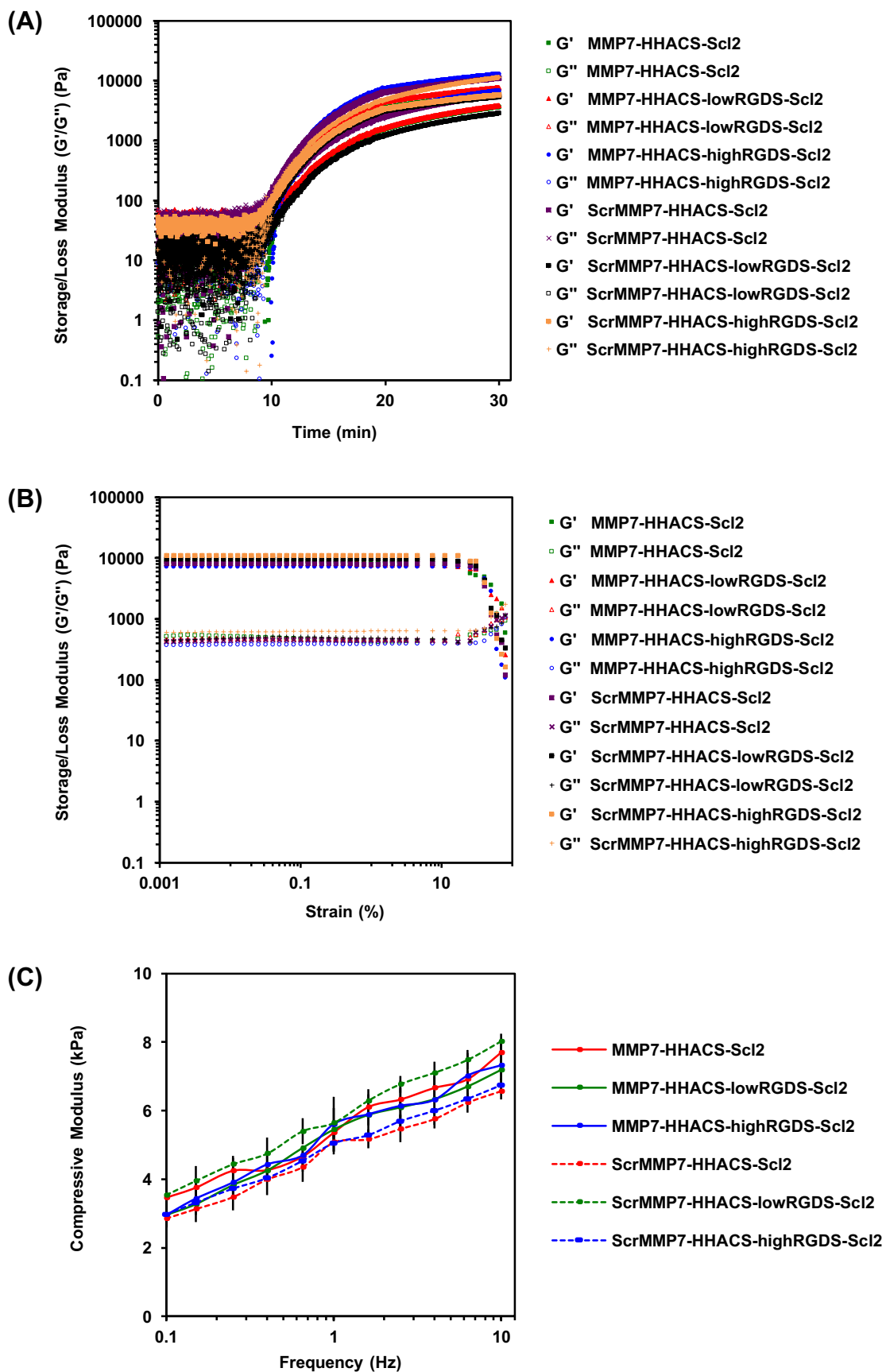


Fig. S7. Mechanical properties of acellular functionalized Scl2 hydrogels. (A) Time to gelation determined at a temperature of 37 °C, angular frequency of 6.28 rad/s, and strain of 0.5% shown as G'

and G'' . (B) Strain sweep at a temperature of 37 °C and an angular frequency of 6.28 rad/s shown as G' and G'' . (C) Dynamic mechanical analysis (DMA) used to determine the elastic modulus in unconfined compression of hydrogels compressed to 10% strain at 0.5% strain/min from 0.1 to 10 Hz and (B). Values represent means \pm SD (n = 3).

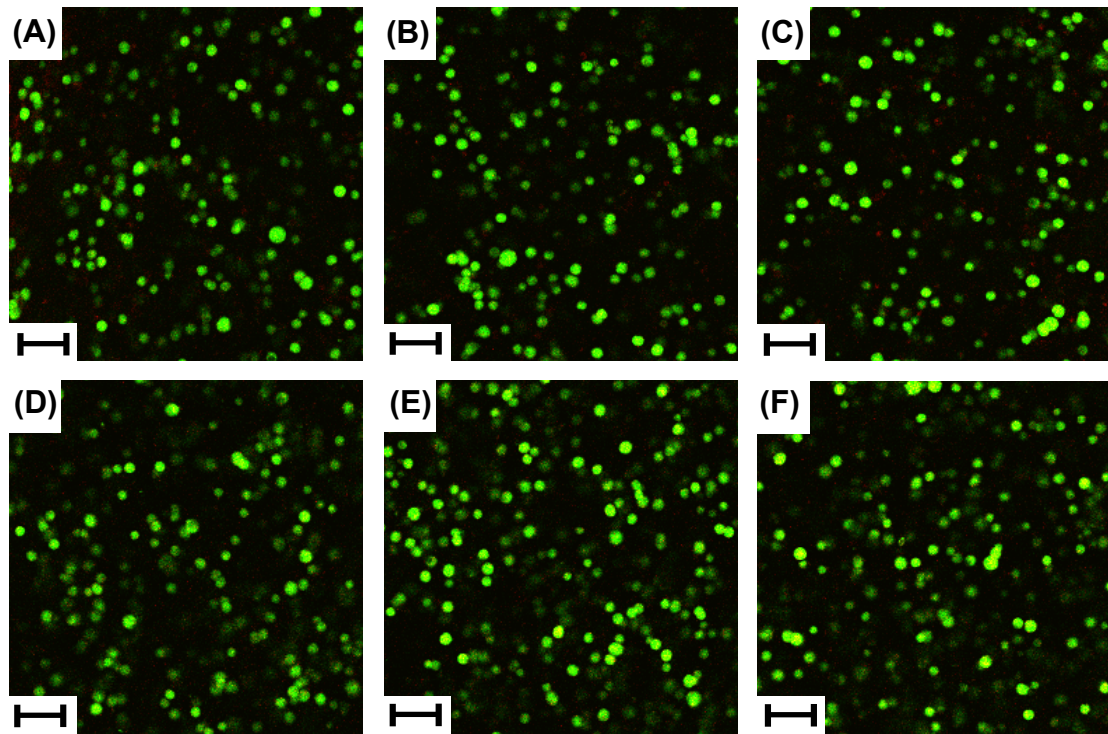


Fig. S8. hMSC viability in Scl2 hydrogels. LIVE/DEAD[®] Viability/Cytotoxicity assay on hMSCs cultured for 6 weeks in hydrogels. Representative confocal images of cells in (A) MMP7-HHACS-Scl2, (B) MMP7-HHACS-lowRGDS-Scl2, (C) MMP7-HHACS-highRGDS-Scl2, (D) ScrMMP7-HHACS-Scl2, (E) ScrMMP7-HHACS-lowRGDS-Scl2, and (F) ScrMMP7-HHACS-highRGDS-Scl2 hydrogels (scale bars are 50 μ m).

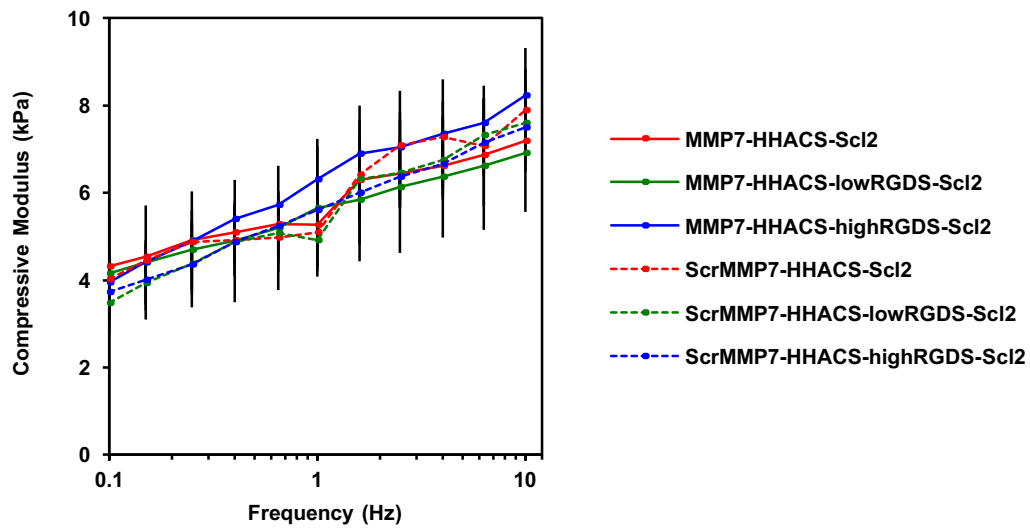


Fig. S9. Dynamic mechanical analysis (DMA). Elastic compression moduli of cell-seeded Scl2 hydrogels compressed to 10% strain at 0.5% strain/min 0.1 to 10 Hz after 6 weeks of culture. Values represent means \pm SD ($n = 3$ for each donor; 3 different bone marrow-derived hMSC donors).

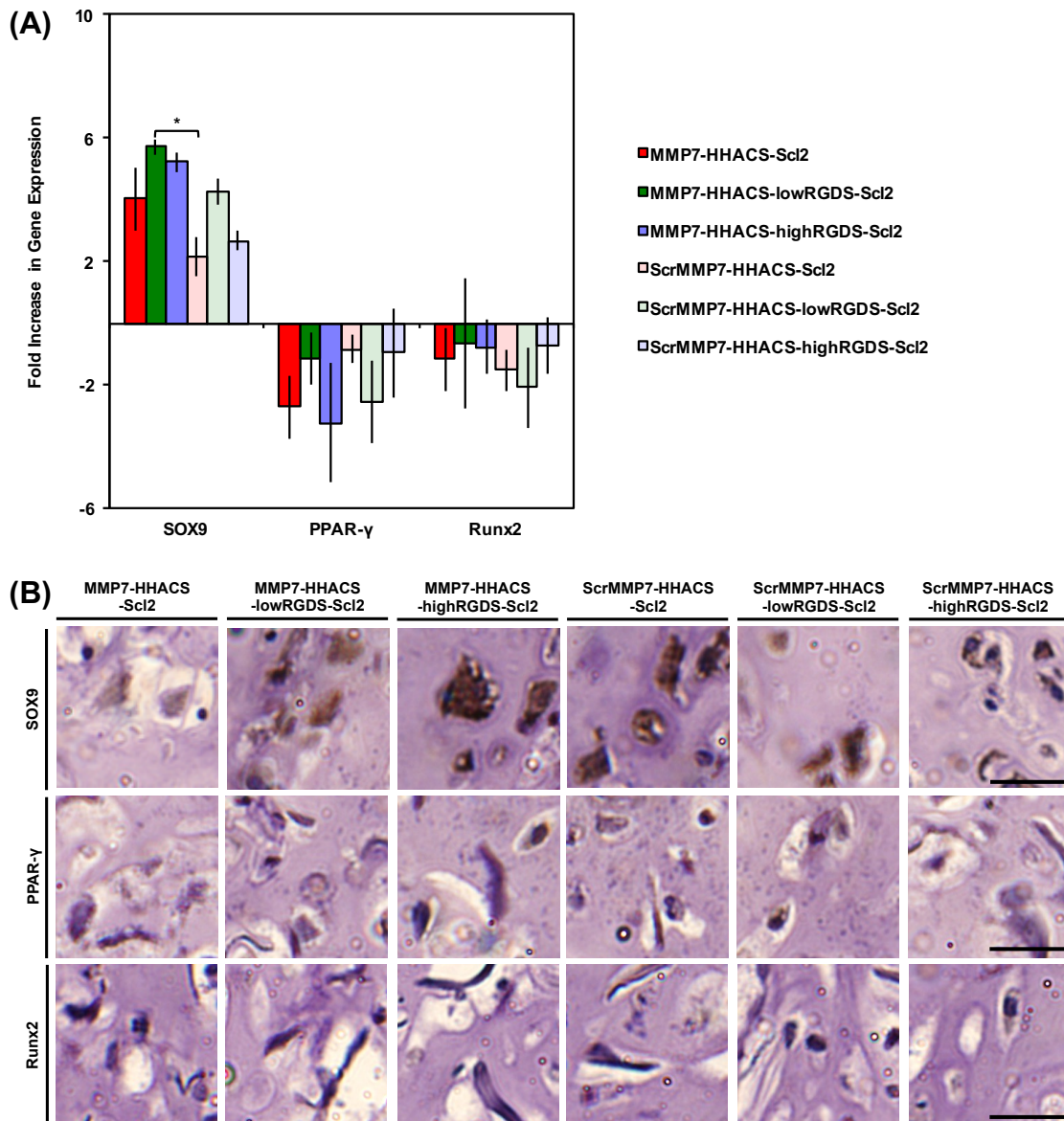


Fig. S10. (A) hMSC gene expression in Scl2 hydrogels. SOX9, Runx2, and PPAR- γ gene expression of hMSCs encapsulated in hydrogels after 2 weeks in culture, as analyzed using the $\Delta\Delta C_t$ method. Data presented as a fold difference relative to undifferentiated hMSCs (calibrator) prior to encapsulation and normalized to GAPDH (housekeeping gene). Values represent means \pm SD. * $p < 0.05$ ($n = 3$ for each donor; 3 different bone marrow-derived hMSC donors). (B) High magnification images showing representative immunohistochemical examination of hMSC-seeded hydrogels after 14 days in culture. Hydrogels are stained for SOX9, PPAR- γ , and Runx2, respectively, from top to bottom. Scale bars are 100 μ m.